ISOLATION, CHARACTERIZATION, AND MECHANISM OF ACTION OF A COMPLEMENT INHIBITOR DERIVED FROM EHRLICH ASCITES TUMOR CELLS

By

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GLOSSARY OF ABBREVIATIONS

E:	Erythrocyte
A:	Antibody
C:	Complement
C1, C2---C9:	Complement components. Horizontal bars over complement components indicate that the components in question are in the biologically active state.
S:	Single site of complement activation.
EAT:	Ehrlich Ascites Tumor
EATC:	Ehrlich Ascites Tumor Cells
RNA:	Ribonucleic Acid

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Tumors possess specific surface antigens and tumor specific antibodies can be shown to exist in many tumor bearing hosts. However, antibodies arising in response to the tumor frequently are noncytotoxic in the presence of complement. Anticomplementary factors in the sera or on the tumor cells may play a role in preventing complement mediated lysis of some tumors. This investigation was initiated to determine if a complement inhibitory substance could be extracted from Ehrlich ascites tumor cells. The anticomplementary effect of Ehrlich ascites tumor cell extracts was investigated using a sheep erythrocyte target cell assay procedure with known amounts of hemolytic antibodies and various complement sources. Experiments were conducted to define the site of action of the inhibitory material. Partial purification of the inhibitory substance was obtained using a combination of phenol extraction and DEAE chromatography. Normal mouse cells were compared with Ehrlich ascites tumor cells to determine if normal mouse cells possess a complement inhibitory material. The

possibility is discussed that the possession of a cell associated complement inhibitor could play a role in the resistance of that tumor to immune rejection.

INTRODUCTION

Complement is a term used to describe a system of nine interacting serum proteins which are normally present in serum as inactive precursors. The complement components are designated C1, C4, C2, C3, C5, C6, C7, C8, and C9, in the order of their reactivity. It is now recognized that the complement system may be activated by two different pathways. The so-called classical pathway is initiated by the interaction of antigen antibody complexes with C1, the first component of complement. As a result of the interaction, C1 is converted to an active form, $C\bar{1}$, which in turn activates and cleaves C4 and C2 (1,2,3). This process generates a second complement complex with enzyme-like activity; the $C\overline{42}$ complex or classical C3 convertase. The alternate pathway of complement action bypasses the early complement components (C1, C2, and C4) and the complement sequence is initiated beginning at C3. The alternate pathway is activated by a number of substances such as inulin, bacterial lipopolysaccharide, zymosan, and a factor derived from Cobra venom. Serum factors, other than complement components, react with the foregoing substances which ultimately leads to the activation of C3 without C1, C4, or C2 participation (4,5). The activation of C3 by either pathway leads to cleavage and activation

of the terminal complement components C5, C6, C7, C8 and C9 (6). Products of the complement system formed in the activation steps are able to mediate a number of biological phenomena such as: increased vascular permeability, leucocyte chemotaxis, enhanced phagocytosis and cell lysis.

The first component of complement is a complex of three different proteins, Clq, Clr, and Cls which exists as a macromolecular complex in vivo (7). Activation of Cl follows the attachment of the component to various substances via the Clq subunit. Antibody is probably the most important activator of Cl. The binding site for Clq on antibody is located on the Fc fragment of IgG and IgM. The acceptor site for Cl is present on the monomer of the immunoglobulin IgG (8). The binding of Cl to monomeric IgG in solution is very unstable (8) and Augener suggests that Cl binding is increased by aggregation (9). Borsos and Rapp, however, showed that it takes a doublet of IgG to initiate complement lysis on the cell surface (10). The binding of antibody to antigen, however, may subsequently reveal or alter additional Cl binding sites on the Fc region so Cl then binds more efficiently to aggregated or fixed immunoglobulin (11,12,13).

Binding of Cl usually leads to Cl activation although both processes can be separated. Modification of tryptophan on IgG with 2-hydroxy-5-nitrobenzyl bromide leads to Clq binding but does not activate Cl (14). The internal activation of Cl occurs by a conformational change of Clq

which induces a change in Clr. Valet and Cooper have shown that the proenzyme Clr is activated in the process so that it acquires enzymatic activity which in turn activates or converts Cls to $C\bar{1}s$ (15). Calcium ions are required to hold the complex Clqrs together and these components can be dissociated in EDTA solution (6,7).

Activation of Cl can also occur by its attachment to various substances such as polyanions and certain lymphocyte and viral membranes. For example, Clq has been shown to react with DNA and RNA (16,17), polyribonucleotides and dextran sulfate (18, 19), and carrageenin (20). Yachnin has reported that certain polynucleotides do not affect complement hemolytic activity (21). Cl activation does not always accompany binding of the component to some substance. Carrageenin interacts with Cl but does not lead to activation of the molecule since Borsos has shown that there is no loss in serum C4 activity when carrageenin is added to serum (20). Ordinarily, attachment of the Cl complex by Clq to immunoglobulin or other substances such as DNA initiates the activation of Clr and ultimately Cls. Once Cl is activated, however, its activity can be inhibited by a normal serum glycoprotein, ClINH (22). ClINH has been shown to inhibit the hemolytic and esterolytic activity of Cls (23,24). Cls, if it is not inhibited by ClINH, in turn activates the next two steps in the complement cascade.

The second phase of complement activation leads to

the formation of two enzymes, C3 convertase ($C\overline{4b2a}$) and C5 convertase ($C\overline{4b2a3b}$). The formation of $C\overline{42}$ enzyme is mediated by the action of $C\overline{1}$. $C\overline{1}$ reacts with C4 and cleaves it into two fragments, C4b, which can interact with C1, and C4a, which is released (3,25). $C\overline{1}s$ next activates and splits C2 into C2a and C2b (26,27). Gigli and Austen have reported that C4 is required for effective activation of C2 by $C\overline{1}s$ (28,29). The activation process then permits the complexing of C4b and C2a to form the enzyme C3 convertase.

The $C\overline{42}$ complex brings about the enzymatic cleavage of C3 into C3a and C3b (30). The C3a part of C3 has been shown to possess anaphalatoxic and chemotactic activities (31,32,33). The fragment C3b has the ability to bind to membranes, and specific receptors for C3b have also been shown to be present on erythrocytes, polymorphonuclear leukocytes, platelets and on B lymphocytes. The binding of C3b has been postulated to play a role in B cell activation as well (34). There are two naturally occurring inhibitors of the fragment C3b in serum. Tamura and Nelson described a substance in guinea pig and rabbit serum that could block C3b activity (35). This material C3bINA, has also been found in human sera (36). Treatment of cell bound C3b by C3bINA releases a fragment C3c or β_1A. A portion of C3 remaining on the cell surface contains the D antigenic determinant of C3 or α_2D (37). The reaction of C3b with C3bINA prevents immune cytolysis

if C3b is on the cell membrane and also inhibits the immune adherence reactivity and the enhanced phagocytic properties associated with C3b. Human sera has also been shown to contain an anaphylatoxin inactivator (AI) which destroys the histamine releasing capacity of C3a as well as C5a (38). The major fragment C3b reacts with the $C\overline{42}$ complex to form a C5 convertase $C\overline{423}$ which activates the membrane attack system. C5 is cleaved in this reaction into two fragments, C5b and C5a (39). C5a has been shown to have chemotactic and anaphylatic activities (32,40,41). The complement components C6 and C7 interact with C5b to form a trimolecular complex that can bind to a membrane site if present (42,43). This complex also has chemotactic activity (44). C8 and C9 react sequentially with the C567 complex to form a large multimolecular complex that, if it is present on the cell membrane, can cause membrane disruption (45). The sequential interaction of the complement components on the cell surface causes ultrastructural lesions to occur on the membrane that can become evident after C5 addition (46,47). However, studies of complement activity on artificial phospholipid membranes indicate that the cell damage might occur through alteration of the membrane lipid (48,49).

The second or alternate pathway of complement can be activated by a munber of substances such as yeast cell walls, inulin, agar, aggregates of immunoglobulins such as IgA, and a factor from cobra venom (40,50,51). The alternate

pathway leads to the direct activation of C3 without the participation of C1, C2, or C4 and then merges with the classical pathway to activate C5 through C9. The alternate pathway is composed of a number of proteins, one of which is C3b which interacts to split C3 into C3a and C3b. These proteins are C3NeF or initiating factor, properdin, C3 proactivation on Factor B, C3 activator or Factor $\overline{B}$ and C3 PA Convertase or Factor $\overline{D}$ (52). These factors have been shown to interact in a sequential fashion, upon activation, to split C3 and generate the membrane attack sequence of complement C5-C9) as well as the biological fragments C3a and C5a.

The recognition of foreign substances in a host usually leads to the elimination of these substances by immune lymphocytes or phagocytic cells or by the concerted effort of specific antibody and complement. If antibodies are made against cell surface antigens, complement could be activated to cause cell lysis or to augment cell mediated immune functions through the activated components and fragments. Tumors possess specific surface antigens, and tumor specific antibodies can be shown to exist in animals and humans with primary tumors (53,54,55,56). However, antibodies arising in response to the tumor frequently are noncytotoxic in the presence of complement (57,58). Tumors may be catagorized by their ability to react with antibody and complement. Leukemias are characterized as being susceptible to cytolysis by antibody and complement,

whereas sarcomas are resistant (59,60,61,62). The reason for the low susceptibility of some tumor cells to the lytic action of antibody and complement may be due to low antigen density on the tumor cells, inaccessability of the humoral factors to the tumor itself, stimulation of non-complement fixing antibodies by the tumor or the presence of complement inhibitors associated with the tumor.

The distribution of tumor antigens might be such that the chances for complement mediated cytotoxicity are remote. Linscott has shown that cells with a low number of antigen sites were not readily susceptible to immune hemolysis even when excess antibody was present (63). This idea is reasonable in view of the report that it takes the random association of about 1000 IgG molecules to generate a doublet needed for complement activation (64,9). However, for mouse leukemia cells, Baker has reported that it takes approximately 1200 IgM molecules to generate an active lytic site which should be more than sufficient to cause lysis if one accepts the sheep E model (65).

Although the density of tumor antigens on the cell surface plays an important role with regard to the effectiveness of cytotoxic antibody and complement, the exposure of the tumor cells to these latter agents is also critical. Serum glycoproteins may form a mucoid barrier that prevents recognition or availability of tumor antigens to antibody and complement (66). For example, plasma from patients immunized with specific tumor antigens was found to be

cytotoxic only when it was injected directly into subcutaneous cancer lesions (67).

The type of antibody made in response to a tumor can vary. Mice have been shown to possess five immunoglobulin classes: IgM, IgA, IgG, IgG_{2a}, IgG_{2b} (68). Only the IgM and IgG_2 fractions have been shown to fix complement (69, 70). Ehrlich ascites tumor cells have been shown to possess bound immunoglobulin in both normal and irradiated C3H mice (71). We have also demonstrated that Ehrlich ascites tumor cells taken from AKR and ICR mice have bound immunoglobulin on their surface (72). Immunoglobulins of IgG_2 complement fixing class have been eluted from chemically induced tumors (73). Hartveit has reported that Ehrlich ascites and Bergen A4 ascites tumor cells, supposedly sensitized with antibody, lysed in the presence of fresh human sera *in vitro*, although fixation of natural antibodies to tumor cells in human sera with the resultant fixation of complement was not ruled out (74). Another report has shown that the availability of complement may be critical since tumor cells sensitized with specific antibody are sensitive to lysis by complement *in vitro*, but may not be lysed when placed in perfusion chambers *in vivo* (75). If specific complement fixing antibodies are present on tumor cells, why then does the host fail to lyse these cells *in vivo*?

Work by Ohanian *et al.* has shown that tumor cells resist lysis even when many C1 molecules have fixed to their surfaces (76). In this report, there was a difference

in the susceptibility of two cell lines of rat hepatoma to complement mediated cytotoxcity when the different cell types were reacted with equivalent amounts of complement components. Cytotoxcity of Maloney virus induced tumors by antibody and complement has been shown to be confined to the G_1 phase of cell growth even though antibody and complement could fix to the cells throughout the cell cycle (77,78). Lerner (77) suggests that there might be some cell cycle changes in the membrane that render it resistant to cytotoxicity or that the ability of the cell to repair damage to the membrane may differ during the cycle. The failure of some tumor cells to be lysed by antibody and complement, therefore, might not be due simply to the unavailability of complement fixing immunoglobulins on the cells.

Anticomplementary factors in the sera of tumor bearers or the tumor itself may play a significant role in preventing complement mediated lysis of some tumors. Evidence that anticomplementary substances are present in sera has been presented in several laboratories. A factor isolated from human and guinea pig sera has been shown to inhibit the sheep EAC$\overline{142}$ intermediate (79). In vitro lysis of mouse tumor cells by peritoneal fluid can be inhibited by normal mouse serum (80). Serum from tumor bearing mice can inhibit lysis of Ehrlich ascites tumor cells by rabbit antibody and guinea pig complement (81). Likewise, lysis of Ehrlich ascites tumor cells by human sera can be inhibited

by ascites fluid (82). Recently, C1 and C3 inactivators have been found in mouse sera as well (83). Dauphinee et al. reported that lysosomal extracts from tumor cells were capable of decreasing the ability of alloantibodies to mediate complement-dependent cytoxicity. The alloantibodies were unaffected by the lysosomal extract in blocking specific killing by alloimmune lymphocytes (84). Cell free Ehrlich ascites fluid has been reported to contain a factor or factors that are capable of blocking immune adherence produced by Ehrlich cells by anti Ehrlich sera. This blocking factor was not tumor specific and was not correlated with the antibody or antibody-antigen complex blocking factors that have been reported (85).

The resistance of tumor cells to the lytic action of antibody and complement might also be due to the presence of a complement inhibitory substance on their surface as well as in serum. There are differences in the sensitivity of red cells from various species to the lytic action of complement (63). Mollison has shown that human erythrocytes are resistant to the action of complement when coated with specific antibody (86). Hoffmann has shown that factors derived from human, rabbit, and guinea pig erythrocytes can inhibit the cytotoxic action of guinea pig and human complement by interfering with C3 convertase (87,88,89). Osther and Linnemann have shown that C1 inactivator is present on human tumor cells in vivo (90). Klein, Harris, and coworkers have reported that Ehrlich ascites

cells might elaborate a factor which makes the cells resistant to cytotoxicity. They also showed in cell fusion studies that the characteristic was genetically controlled and dominant (91).

The relative susceptibility of a tumor to the action of complement might be due, in part, to the association of a complement inhibitor with that tumor. The association of a complement inhibitory substance would also lower the cell mediated responses that are complement dependent such as increased phagocytosis and chemotoxis of leukocytes. A tumor cell associated complement inhibitor might correlate with the persistance of certain tumors.

This investigation was initiated in an attempt to answer the question, "Do tumor cells possess a factor or factors that can interfere with complement mediated cytotoxicity?" A sheep erythrocyte target cell assay procedure was utilized using known amounts of hemolytic antibodies and various complement sources. In this way, anticomplementary effects would be easily detected. In subsequent studies a tumor cell target system was utilized.

Ehrlich ascites tumor cells were chosen as a model since they are easily obtainable and growth is not restricted to a particular mouse strain (91). Extracts from Ehrlich ascites tumor cells have been found to inhibit complement mediated lysis of sensitized sheep red blood cells. Experiments were conducted to define the site of action of the inhibitory material, and partial purification of the

inhibitory material has been obtained. Normal mouse cells were compared with Ehrlich ascites tumor cells to determine if normal mouse cells possess a complement inhibitory material. The possession of cell associated complement inhibitory substance could have a relationship with persistence of that tumor in a given host.

MATERIALS AND METHODS

Solutions

Stock Veronal buffered saline (Stock VBS). A stock five times concentrated sodium chloride-Veronal buffer solution was prepared according to the method described by Mayer (92).

Veronal buffer with $CaCl_2$ and $MgCl_2$ (VB^{++}). This solution was prepared by mixing 100 ml of Stock VBS, 2.5 ml of 0.2M $MgCl_2$, 2.5 ml of 0.03M $CaCl_2$ with enough distilled water to bring the volume to 500 ml.

Gelatin Veronal buffer ($GVB^{=}$). This solution was prepared by mixing 100 ml of Stock VBS and 25 ml of 2 percent (w/v) gelatin with enough distilled water to bring the volume to 500 ml.

Gelatin Veronal buffer with $CaCl_2$ and $MgCl_2$ (GVB^{++}). This solution was prepared by mixing 100 ml of Stock VBS, 25 ml of 2 percent gelatin, 2.5 ml of 0.2M $MgCl_2$, 2.5 ml of 0.03M $CaCl_2$ with enough distilled water to bring the volume to 500 ml.

Gelatin Veronal buffer with double concentrations of gelatin, $CaCl_2$ and $MgCl_2$ ($2XGVB^{++}$). $2XGVB^{++}$ was prepared by mixing 100 ml of Stock VBS, 50 ml of 2 percent gelatin, 5 ml of 0.2M $MgCl_2$, 5 ml of 0.03M $CaCl_2$ with distilled

water to bring the volume to 500 ml.

Dextrose Gelatin Veronal buffer ($DGVB^{++}$). $DGVB^{++}$ was prepared by mixing equal volumes of 5 percent (w/v) D-Glucose and $2XGVB^{++}$.

0.04M EDTA-$GVB^{=}$. This solution was prepared by mixing equal volumes of 0.08M isotonic stock trisodium ethylenediaminetetraacetate (EDTA) solution (pH 7.5) and $GVB^{=}$.

Phosphate buffered saline (PBS). Sodium chloride solution (3M) was diluted with 0.005M, pH 7.5 potassium phosphate buffer. The solution was adjusted to an ionic strength of 0.15μ with water. A conductivity bridge (Model RC 16 B2) was used to measure the electrical conductance of the solutions and ionic strength was estimated by comparison with conductance values from a standard sodium chloride calibration curve.

Complement, Human (HuC). Fresh human blood was obtained from the Gainesville Plasma Corporation, Gainesville, Florida. The blood was allowed to clot at room temperature for about 60 min, and the serum was separated by centrifugation at 500G at 0°C. The sera were collected and stored at -70°C.

Complement, Rabbit (RC). Fresh rabbit blood was obtained from New Zealand White strain animals by cardiac puncture. The blood was allowed to clot at room temperature for about 30 min and the serum was separated at 500G at 0°. The sera were collected and stored at -70°C.

Complement, Mouse (MC). Mouse blood was obtained by puncture of the retroorbital socket with a pasteur pipette. The blood was transferred to test tubes and allowed to clot at 0°C. The sera were collected and stored at -70°C.

Complement Components. Purified guinea pig C1, C2, and C4 were prepared according to Nelson et al. (93) and Ruddy and Austen (36,94). Purified guinea pig C3, C5, C6, C7, C8, and C9 were purchased from Cordis Laboratories (Miami, Florida). Purified Human C1q was prepared by method of Yonemasu and Stroud (95).

Erythrocytes. Sheep blood was taken by venipuncture from a single animal maintained at the Animal Research Laboratory of the J. Hillis Miller Health Center (Gainesville, Florida). One volume of blood was mixed with an equal volume of sterile modified Alsever's solution (92), and the blood was stored at 4°C for up to 3 weeks.

Antibody sensitized sheep E (EA). Rabbit anti-sheep erythrocyte (E) stromata was obtained from Cordis Laboratories (Miami, Florida). Sensitization was performed as recommended by the manufacturer.

Complement component intermediates (cell intermediates). Sheep E in various states of complement component fixation were used in this study. All complement components listed are guinea pig components unless indicated otherwise. $EAC\overline{1}$, EAC4, $EAC\overline{14}$, and $EAC\overline{142}$ were prepared by the methods described by Borsos and Rapp (96). $EAC\overline{14235}$ and $EAC\overline{142356}$

were prepared by the method described by Hoffmann (88). The intermediate $EAC\overline{1_{Hu}423567}$ was purchased from Cordis Laboratories (Miami, Florida). $EAC\overline{1_{Hu}42356789}$ was prepared by mixing limiting amounts of C8 and C9 with $EAC\overline{1_{Hu}423567}$.

Animals. Male ICR mice were purchased from Flow Laboratories. Male DBA/2J and C57Bl/6J were purchased from Jackson Laboratories (Bar Harbor, Maine). Male, New Zealand White rabbits were obtained from Animal Research Laboratories of the J. Hillis Miller Health Center (Gainesville, Florida).

Tumor Cells. Ehrlich ascites tumor cells (EATC) were obtained from Dr. Paul Klein (University of Florida, Department of Pathology) and maintained by weekly passage of 10^6 cells into ICR mice. P815 and E1-4 tumor cells were also obtained from Dr. Paul Klein.

Preparation of EATC Extracts. Ehrlich ascites tumor cells were harvested from the peritoneal cavities of mice seven days after inoculation with tumor cells. The cells were washed with PBS at 50G for 10 min until no red cells were detectable. A crude membrane fraction of EATC was prepared by freeze-thawing the cells in 10 volumes of distilled water. The insoluble portion was extensively washed with ice cold distilled water until the supernate was clear. The insoluble portion (1000G, 10 min pellet) was then used as a crude membrane fraction.

Three different methods of extraction were used.

For the first method, the crude membrane fraction of EATC was suspended in an equal volume of 0.005M potassium phosphate, pH 7.5 and extracted with n-butanol using the method described by Hoffmann for extraction of human red cell stromata (87). The second method consisted of extraction of crude EATC cell membranes three times with 0.5M sodium chloride buffered at pH 7.5 with 0.005M potassium phosphate according to the methods that have been previously described (87). The third method involved extraction of intact EATC. Washed packed tumor cells were shaken slowly overnight on Burrell Wrist Action Shaker (Burrell Corporation, Pittsburgh, Pennsylvania) at 4°C with 3 volumes of various concentrations of sodium chloride buffered with 0.005M potassium phosphate at pH 7.5. The cell suspensions were centrifuged first at 500G for 10 min to sediment the cells and then at 10,000G for 30 min. The supernates were dialyzed against PBS overnight at 4°C.

Inhibition of immune hemolysis by cell extracts. Extracts from Ehrlich ascites tumor cells and from other sources were tested for the ability to inhibit whole complement activity. Extracts were serially diluted in PBS, and 0.2 ml of each dilution was added to a series of test tubes to which 0.2 ml of EA, at a concentration of 1 X 10^8/ml, and 0.6 ml of complement at a concentration to give approximately 75 percent hemolysis, were added. In a control tube, 0.2 ml of PBS was added in place of

the extract. After 60 min incubation at 37°C with continuous shaking, 2.0 ml of ice cold PBS were added to each tube. The mixtures were centrifuged and the optical densities of the supernates were determined at a wavelength of 414nm. The inhibition of hemolysis was calculated for each dilution of extract used.

Complement fixation by tumor cell extracts. Ten volumes of guinea pig complement were adsorbed 3 times at 0°C with 1 volume of washed, packed EATC. One volume of adsorbed complement and 1 volume of tumor cell extract were added to a test tube and incubated at 30°C for 15 min. The guinea pig complement was then titrated in the standard complement titration assay described by Mayer (92).

Inhibition of hemolytic antibody titration by tumor cell extracts. In order to determine if extracts from EATC contained any shared antigens with sheep red cell stroma, a hemolytic antibody titration was performed. Equal volumes of EATC extracts and rabbit anti-sheep E stromata (Cordis Laboratories, Miami, Florida) were incubated for 15 min at 30°C and at 0°C for 15 min. A control consisted of incubating equal volumes of PBS and anti-sheep E for the prescribed length of time. The antibody was titered using limiting amounts of complement as described by Mayer (97).

Decay of $\overline{SAC1}42$ by tumor cell extracts. To determine if there was any influence on the rate of decay of

$SAC\overline{142}$, extracts from EATC were incubated with $EAC\overline{142}$ and assayed according to the method described by Hoffmann (88).

Decay of $SAC\overline{1}4$ by tumor cell extracts. To determine if there was any influence on the stability of $SAC\overline{1}4$, extracts from EATC were incubated with $EAC\overline{1}4$ and assayed according to the method described by Hoffmann and Etlinger (89).

Hemolytic inhibition assays using sheep E, EA, and various cell intermediates. Analysis of inhibition at the cellular level was examined using the methods described by Hoffmann(88). This method consisted of incubating one volume of the intermediate to be tested with an equal volume of EATC extract at 30°C for 15 min. The cells were washed two times at 0°C and resuspended to a concentration of 1 X 10^8/ml and the necessary reagents were then added, as described, in concentrations sufficient to cause between 50 and 80 percent hemolysis.

Generation of $EAC\overline{142}$ in the presence of EATC extracts. In order to determine if tumor cell extracts had any effect on $SAC\overline{142}$ generation, Tmax experiments were performed. Tmax is the time required for the generation of the maximum number of $SAC\overline{142}$ per cell (98). Equal volumes of $EAC\overline{1}4$ and tumor cell extracts were incubated at 30°C for 15 min. A control tube consisted of PBS instead of tumor extract. After incubation, the cells were washed two times and resuspended to a concentration of 1 X 10^8 cells per ml. The cells were prewarmed to 30°C and a

Tmax experiment was performed as described by Borsos et al. (98). The absorbancies of the supernates were read at a wavelength of 414nm, and the data plotted as number of $SAC\overline{142}$ per cell (Z number) versus time (99).

Cl fixation and transfer. The number of Cl molecules bound to antibody-antigen complexes can be measured by the Cl fixation and transfer test described by Borsos and Rapp (64). Their procedure was used here in an attempt to quantitate the number of Cl molecules fixed to EA which had been previously treated with tumor cell extract. This Cl test was carried out in different stages. Equal volumes of EA (1 X 10^8/ml) and tumor cell extract were incubated at 30°C for 15 min, washed twice with $DGVB^{++}$ and resuspended to 1 X 10^8 cells/ml in $DGVB^{++}$. A control consisted of treating EA with PBS. Equal volumes of EA treated with tumor cell extract (EA_T) or EA treated with PBS (EA_C) and Cl were incubated at 30°C for 10 min in $DGVB^{++}$. The cell mixtures were washed twice with $DGVB^{++}$, resuspended in GVB^{++} at a concentration of 1 X 10^8/ml, 5 X 10^7/ml, 1 X 10^6/ml, 5 X 10^5/ml, and 1 X 10^5/ml. One volume of each cell concentration was added to one volume of EAC4 to permit transfer of Cl from $EAC\overline{1}$ to EAC4. The cells were incubated at 30°C for 10 min. C2 and C-EDTA were then added in relative excess as described.

In another experiment, $EAC\overline{1}$ were treated with tumor cell extract or with PBS at 30°C for 15 min. The cells

were washed twice in $DGVB^{++}$ and resuspended in GVB^{++} at a cell concentration of 1 X 10^8/ml. The amount of C1 capable of transfer was then measured in the C1 transfer test described above.

Consumption of C4 by tumor cell extract treated $EAC\bar{1}$. In order to measure the activity of C1 on the intermediate $EAC\bar{1}$ that had been treated with tumor cell extract, a C4 consumption experiment was performed. $EAC\bar{1}$ at a concentration of 1 X 10^8/ml were generated as described (96) and treated with tumor cell extract at 30°C for 15 min, washed twice and resuspended to a cell concentration of 5 X 10^8/ml. $EAC\bar{1}$ treated with tumor cell extract are designated $EAC\bar{1}_T$. Control cells treated with PBS are designated $EAC\bar{1}_C$.

$EAC\bar{1}$ cells were separately mixed with an equal volume of C4. The mixtures were incubated at 30°C for 10 min. The cells were centrifuged at 500G for 5 min, the supernatent fluids were serially diluted in $DGVB^{++}$, and 0.2 ml of each dilution was added to 0.2 ml of $EAC\bar{1}$ that had not been treated with extract or PBS. The mixtures were incubated at 30°C for 20 min. An equal volume of C2 (0.2 ml) was added to each tube and the mixtures incubated for an additional 20 min at 30°C. C-EDTA (0.4 ml) was added to each tube and the mixtures were incubated at 37°C for 60 min. After the incubation period, 2.0 ml of ice cold PBS were added to each tube. The reaction mixtures were centrifuged at 500G for 5 min at 0°C. The absorbancy of the

supernates was read at a wavelength of 414nm, and the data plotted as described by Rapp and Borsos (99).

Titration of individual complement components. In order to determine if extracts from tumor cells had any inhibitory effect on individual complement components, whole guinea pig sera or each component to be examined were incubated with extract or buffer at 30°C for 15 min. The sera were then titrated for the component in question or the individual component was titrated as described (93). In each case, the dilution of tumor cell extract used in the initial incubation was inhibitory in the hemolytic assay, but the inhibitory effect of the extract was diluted out in the titration range of the component being tested.

Ammonium sulfate fractionation of crude tumor cell extract. Tumor cell extracts were initially fractionated by using ammonium sulfate. A saturated ammonium sulfate solution was added to extracts of EATC to bring the final concentration of ammonium sulfate to 20, 40, 60, and 80 percent saturation. The mixtures were incubated at 0°C for 1 hr and centrifuged at 10,000G for 30 min. The supernates were transferred directly to dialysis tubing and dialyzed overnight versus PBS with two changes of the dialysis buffer. The pellets obtained after centrifugation were redissolved in a minimal amount of PBS, transferred to dialysis tubing and dialyzed overnight as above. The dialyzed fractions were then tested for inhibitory activity using the immune hemolysis inhibition test.

Gel filtration and ion exchange chromatography. For gel filtration, crude tumor cell extracts were applied to a 2.5 X 80/cm column of G-200 Sephadex (Pharmacia Fine Chemicals, Piscataway, New Jersey) which had been equilibrated with PBS. The column was eluted with PBS and fractions were assayed for the ability to inhibit whole complement mediated lysis of EA.

For Bio-Gel A-1.5m separations, partially purified EATC extracts were applied to a 1.5 X 80/cm column of Bio-Gel A-1.5m (Bio-Rad Laboratories, Richmond, California) which had been equilibrated with PBS. The column was eluted and assayed as described for G-200 Sephadex.

For DEAE chromatography, standard capacity Cellex D (.67meq/gram) was prepared according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, California). Tumor cell extract was adjusted to an ionic strength of 0.3 pH 7.5 (0.005M potassium phosphate - sodium chloride buffer) and applied to a 4.0 X 40cm DEAE column that had been equilibrated to an ionic strength of 0.3 pH 7.5. After application of the extract, the column was washed with the initial buffer and fractions were collected until the absorbancy at 280nm was near zero. A linear salt gradient buffered with 0.005M potassium phosphate pH 7.5, was then initiated and fractions were collected as described in the results section. The fractions were adjusted to 0.15 ionic strength using distilled water and assayed for the ability to inhibit complement mediated lysis of EA.

Enzyme sensitivity. Tumor cell extracts were subjected to various enzyme treatments in order to determine which class of compounds was responsible for the inhibitory activity. Deoxyribonuclease, ribonuclease free, (Worthington Biochemical Corporation, Freehold, New Jersey) was used at a concentration of 100μg/ml in VB^{++}. One volume of deoxyribonuclease was added to four volumes of tumor cell extract. Controls consisted of mixing one volume of enzyme with four volumes of PBS and by mixing one volume of VB^{++} with four volumes of tumor cell extract. The mixtures were incubated for 30 min at 25°C and each mixture was titrated for the ability to inhibit whole complement mediated lysis of EA. Ribonuclease A (Worthington Biochemical Corporation, Freehold, New Jersey) was used at a concentration of 100μg/ml in VB^{++} and Ribonuclease A, type III (Sigma Chemical Company, St. Louis, Missouri) was used at a concentration of 1.0mg/ml in VB^{++}. One volume of ribonuclease and four volumes of tumor cell extract were mixed together and incubated for 30 min at 37°C. The controls used and the inhibition test are described above. One volume of trypsin, type III (Sigma Chemical Company, St. Louis, Missouri) at a concentration of 2.5mg/ml was mixed with four volumes of tumor cell extract. The controls are the same as indicated for deoxyribonuclease. The mixtures were incubated for 60 min at 37°C. One volume of lima bean trypsin inhibitor (Sigma Chemical Company, St. Louis, Missouri) at ten times equimolar amounts of trypsin was added to each tube to

stop trypsin enzymatic activity. The mixtures were then titrated for inhibitory activity in the whole complement inhibition test. The protease, obtained from Streptomyces griseus, was purchased from Miles Laboratories (Kanakee, Illinois) and was used as an insolubilized enzyme in a 2.5 ml syringe type column. The void volume of the column was approximately 2 ml. The column was equilibrated with VB^{++} and the buffer was allowed to drain to the top of the column. One volume (1.0 ml) of tumor cell extract was applied and allowed to drain to the top of the column. The column was closed and incubated for 60 min at 25°C. After incubation, 2 ml of VB^{++} was applied to the column to elute the tumor cell extract. The dilution of extract on the protease column was approximately twofold. The extract was then compared with a 1:2 dilution of untreated extract for its ability to inhibit complement mediated lysis of EA. Controls consisted of adding one volume of PBS to the column as described for the tumor extract.

Streptomycin sulfate fractionation. Streptomycin sulfate was purchased from Sigma Chemical Company, St. Louis, Missouri. The fractionation of the crude tumor cell extract with streptomycin sulfate was performed according to the procedure of Stern and Mehler (100). Three columes of a 6 percent streptomycin sulfate solution were added, over a 10 min period with stirring, to 10 volumes of crude extract. The precipitate was allowed to coagulate and settle overnight. The mixture was centrifuged at

500G for 10 min and the supernate was dialyzed overnight versus PBS. The supernate was then assayed for its ability to inhibit complement mediated hemolysis of EA.

Heat sensitivity of tumor cell extracts. Crude and partially purified Ehrlich ascites tumor extracts were placed in a 56°C water bath. At timed intervals, samples were taken and transferred to an ice bath. Each sample was then assayed for its ability to inhibit lysis of EA by whole guinea pig complement.

Sucrose gradient centrifugation. Two-tenth ml samples were applied to a preformed buffered sucrose gradient(ionic strength = 0.05, pH 7.6, 5 percent to 20 percent w/v sucrose) of 5.0 ml. The tubes were centrifuged at 38,000 rpm for 5 hr in a Sw-39 rotor of a Model L-2 Beckman Ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California). After centrifugation, the mixture was pumped on a Buchler Polystaltic Pump (Buchler Instruments, Fort Lee, New Jersey) at a flow rate of 0.6 ml/min from the bottom of each tube through a Gilford Model 2400 Spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) continuously monitored at 260nm and recorded. Fractions were collected and assayed for inhibition of immune hemolysis.

Viability of EATC and release of complement inhibitory material. One volume of washed intact EATC was incubated with 3 volumes of PBS at 4°C with mild shaking. At timed intervals, 5 ml samples were taken and the viability of the cells determined by trypan blue exclusion (62) on 0.2 ml samples. The remainder of the sample was

centrifuged at 500G for 10 min to sediment the cells and the supernate was centrifuged at 10,000G for 30 min. The ability of the supernate to inhibit complement mediated hemolysis of EA was titered.

Extraction of normal mouse tissue cells and whole tumor cells for nucleic acid. One volume of liver and spleen cells from ICR mice and Ehrlich ascites tumor cells was suspended in one volume of PBS. The cells were frozen and thawed rapidly 3 times. The cells were then placed at 0°C and extracted using a modification of the phenol extraction procedure (101). One volume of 88 percent aqueous phenol (Fisher Scientific, Pittsburgh, Pennsylvania) was added to 1 volume of cells at 0°C with constant stirring. After 10 min, the solution was centrifuged at 10,000G for 10 min. The aqueous phase was carefully removed and transferred to another tube. An equal volume of PBS was added to the phenol phase and the mixture was stirred at 0°C for 10 additional min and centrifuged. The second aqueous layer was removed and added to the first aqueous phase. Two and one half volumes of ice cold 95 percent ethanol were added to the pooled aqueous layer, stirred briefly, and the mixture was stored overnight at -20°C. The mixtures were centrifuged at 1000G for 30 min at 0°C. The supernate was discarded and the pellet was washed 2 additional times with 95 percent ethanol. After the last washing, the supernate was discarded and the tube was allowed to drain dry briefly. A small volume of PBS was added to the pellet. The mixture was

stirred carefully with a glass rod to dissolve the pellet. Two and one half volumes of 95 percent ethanol were added to the dissolved pellet and the procedure repeated as before. After washing the second precipitate, the precipitate was dissolved in PBS, transferred to dialysis bag and dialyzed overnight versus 20 volumes of PBS at 4°C. The dialyzed extract was then frozen at -70°C.

Inhibition of immune hemolysis by RNA. RNA from Escherchia coli and yeast were used to determine if they were capable of inhibiting guinea pig complement mediated lysis of EA. Total E. coli RNA was obtained from Dr. James Preston (University of Florida, Department of Microbiology). Yeast RNA was purchased from Sigma Chemical Company (St. Louis, Missouri). The RNA was resuspended in PBS and titrated for its ability to inhibit immune hemolysis.

Extraction of tumor cell extract with phenol. Crude extracts obtained by incubating whole EATC with 0.15M NaCl pH 7.5 were further extracted with aqueous phenol as outlined above.

Ascitic fluid extraction. Cell free ascitic fluid obtained from harvesting EATC from ICR mice was also subjected to phenol extraction as outlined above.

Extraction of P815 and EL4 tumors. The tumor lines P815 and EL4 were obtained from Dr. Paul Klein (University of Florida, Department of Pathology). P815 cells were maintained by weekly passage of 10^6 cells into DBA/2J mice and the EL4 tumor line was maintained in C57Bl/6J. The

tumors were harvested after one week and washed extensively with PBS as outlined for EATC. Washed, packed tumor cells were shaken overnight at 4°C with 3 volumes of PBS. The cell suspensions were centrifuged first at 500G for 10 min to sediment the cells and then at 10,000G for 30 min. The supernates were then assayed for their ability to inhibit lysis of sensitized sheep erythrocytes with guinea pig complement that had previously been adsorbed with each tumor line investigated.

Extraction of liver and spleen cells. Spleen and liver cells obtained from non-tumor bearing ICR mice were extracted with PBS as outlined for P815 and EL4 tumors.

Cytotoxicity tests. Antisera to EATC were prepared as outlined previously (73). EATC were harvested from ICR mice and washed extensively, first in PBS and then in $DGVB^{++}$. The cells were standardized to 5 X 10^6 cells/ml. In the first set of experiments, 1 volume of EATC (5 X 10^6 cells/ml) was incubated for 15 min at 30°C with an equal volume of ribonuclease A (Worthington Biochemical Corporation, Freehold, New Jersey). The ribonuclease was used at a concentration of 200 ug/ml. After incubation, the cells were centrifuged, washed 2 times with $DGVB^{++}$ and restandardized to 5 X 10^6 cells/ml. A control consisted of treating EATC with $DGVB^{++}$. One volume (0.025 ml) each of the cell suspensions was added to separate wells of plastic microtiter plates (Cooke Engineering Company, Alexandria, Virginia). One volume (0.025 ml) of $DGVB^{++}$ was added to

each well, as well as one volume (0.025 ml) of anti-EATC sera and one volume (0.025 ml) of a 1:20 dilution of guinea pig serum which had been previously adsorbed with EATC. The plates were incubated for 60 min at 37°C with shaking. The test was read by placing the microtiter plates in ice, adding 0.025 ml of trypan blue solution to each well and counting the proportion of stained (dead) cells in a hemocytometer. The trypan blue solution was prepared by dissolving 1.0gm of trypan blue in 100 ml of distilled water. At the time of use, 4 parts of this solution were diluted with one part of a 4.25 percent NaCl solution.

In another set of experiments, EATC were not pretreated with ribonuclease but the enzyme was substituted for $DGVB^{++}$ in the reaction mixture outlined above.

Extracts from EATC were also assayed for their ability to inhibit cytotoxicity of EATC by antibody and complement. One volume (0.025 ml) of tumor cell extract was incubated with an equal volume of PBS and serially diluted in plastic microtiter plates. Ehrlich ascites tumor cells at a concentration of 5×10^6 cells/ml were incubated with 1 volume of anti-Ehrlich antiserum (1:5 dilution) at 37°C for 10 min and at 0°C for 15 min. The cells were centrifuged and washed 2 times with $DGVB^{++}$ and restandardized to 5×10^6 cells/ml. One volume (0.025 ml) of the sensitized EATC was then added to each dilution extract. One volume (0.025 ml) of EATC was also added to control wells containing 0.025 ml PBS. Two volumes (0.05 ml) of guinea pig serum diluted 1:40 in

$DGVB^{++}$ were added to each well. The plates were incubated at 37°C for 60 min. After incubation, the dead cells were scored as before. In each experiment, each test was performed in triplicate.

Immunodiffusion analysis and reaction of Clq with tumor cell extracts. Clq was tested for its ability to agglutinate gamma globulin coated latex particles (Hyland Laboratories, Los Angeles, California) according to the method of Ewald and Schubart (102). Tumor cell extracts were serially diluted in PBS. Clq was diluted in glycine-saline buffer as described by Ewald and Schubart. One volume of each dilution of tumor cell extract was mixed with an equal volume of each dilution of Clq. The mixtures were incubated for 15 min at 30°C. After incubation, the mixtures were titered for their ability to agglutinate gamma globulin coated latex particles. Ouchterlony double diffusion was carried out in 0.6 percent agarose in .005M potassium phosphate containing 0.01M EDTA, relative salt concentration (RSC) = 0.09, pH 7.2 as supporting medium according to the method of Agnello et al. (16). Anti-human sera and anti-human IgG, IgM, and IgA were purchased from Hyland Laboratories (Los Angeles, California). Anti-human Clq was purchased from Behring Diagnostics (Somerville, New Jersey).

Clq purified according to the method of Yonemasu and Stroud (95), did not contain any detectable IgG, IgM, or IgA by Ouchterlony double diffusion. Clq reacted with

anti-human sera and anti-Clq with a band of identity. There was a trace contaminant, however, when Clq was reacted against anti-human sera. The purified Clq was then tested for its ability to react with dilutions of tumor cell extract in Ouchterlony double diffusion, according to the method of Agnello (16).

RESULTS

Extraction of complement inhibitory material from EATC with various salt solutions. Washed packed tumor cells were incubated overnight at 4°C in various concentrations of buffered sodium chloride solutions in order to determine if a complement inhibitory substance would be released from Ehrlich ascites tumor cells. The extracts were assayed for their ability to cause inhibition of immune hemolysis using a limiting amount of guinea pig complement which had been adsorbed previously with EATC. The inhibitory effects of the various salt extracts of EATC are shown in Figure 1. Extraction of EATC with buffered 0.15M sodium chloride (PBS) yielded the highest relative amount of inhibitory material using the parameters measured.

Inhibition of immune hemolysis by extracts from whole tumor cells and crude membranes of EATC. Since extracts from human erythrocyte membranes have been shown to inhibit complement activity (87), extracts from crude membrane fractions of EATC were compared with the PBS extract from whole tumor cells for their inhibitory effects on the lysis of EA by guinea pig complement. In each case, guinea pig serum which had been adsorbed with EATC was used as the source of complement. Figure 2 shows the inhibition of lysis of EA caused by the tumor cell extracts. Since the

Figure 1. Inhibition of immune hemolysis by various salt extracts of Ehrlich ascites tumor cells. The curves represent the relative salt concentration of the solutions used for the extraction of EATC. All extracts were adjusted to an ionic strength of 0.15M before assaying. The ionic strengths (μ) of the solutions used were (○—○), 0.05μ; (□—□), 0.15μ; (△—△), 0.2μ; (●—●), 0.3μ; (■—■), 0.4μ; (▲—▲), 0.5μ.

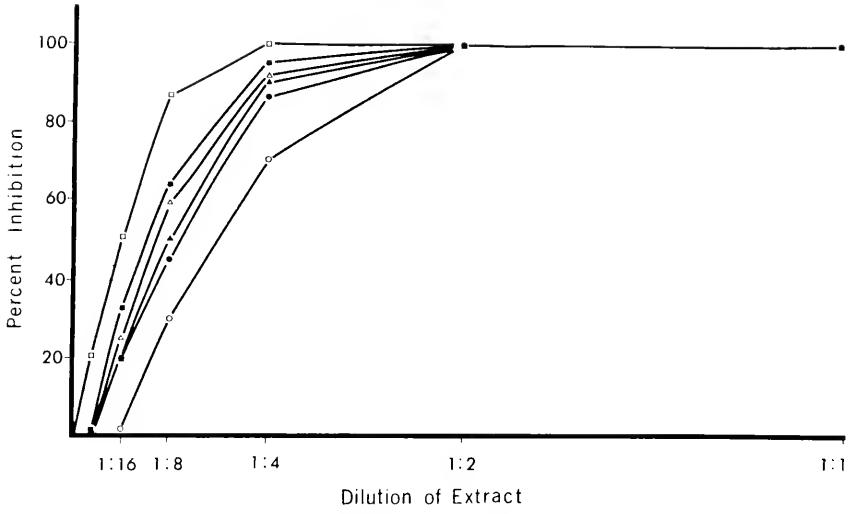
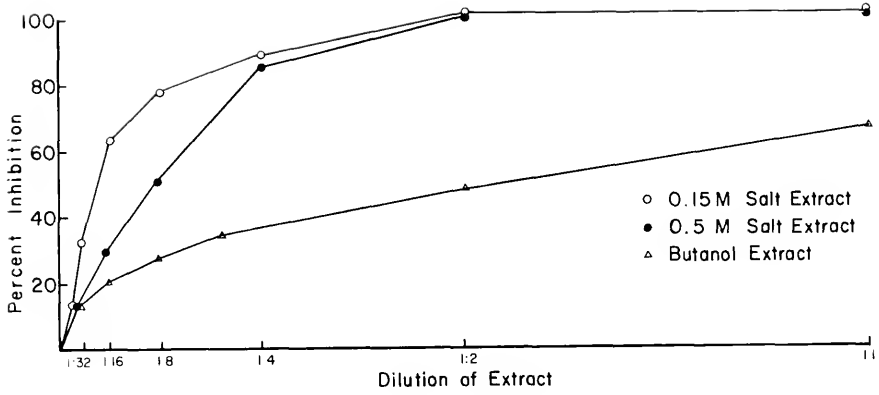

100
80
60
40
20
Percent Inhibition
1:16
1:8
1:4
1:2
1:1
Dilution of Extract

Figure 2. Inhibition of immune hemolysis by EATC extracts. Extracts obtained with the 0.15M salt solution were obtained from washed, packed tumor cells. The extracts obtained with 0.5M NaCl and butanol were obtained by treating a crude membrane fraction of EATC.


100
80
60
40
20
Percent Inhibition
1:32
1:16
1:8
1:4
1:2
1:1
Dilution of Extract
0.15M Salt Extract
0.5 M Salt Extract
Butanol Extract

extractions of intact tumor cells with PBS resulted in relatively good yields under the mildest conditions, the PBS extract was used in subsequent experiments unless otherwise noted.

Effects of EATC extracts on rabbit, mouse, and human complement. Since extracts from EATC were capable of inhibiting the lysis of EA by guinea pig serum, the extracts were tested to determine if they could inhibit complement from other sources. Rabbit, mouse, and human sera were adsorbed with Ehrlich ascites tumor cells before use. A 0.15M sodium chloride extract from EATC was diluted and tested for its ability to inhibit immune hemolysis with various complement sources. The inhibition of guinea pig complement by this extract is shown in Figure 3. The inhibitory capacity of the extract was then tested in the other complement systems. Figure 4 shows the inhibition of rabbit complement by this extract. It appears that the tumor cell extract inhibits rabbit complement to a slightly greater extent than guinea pig complement. A maximun of 60 percent inhibition of mouse complement was obtained by this EATC extract as shown in Figure 5. It is not clear why mouse serum could not be inhibited beyond 60 percent. Finally, Figure 6 shows the relative inhibition of human complement caused by the extract.

Complement fixation by tumor cell extracts. The inhibition of whole complement activity caused by tumor cell extract could have conceivably been due to complement

Figure 3. Inhibition of guinea pig complement activity by Ehrlich ascites tumor cell extracts.

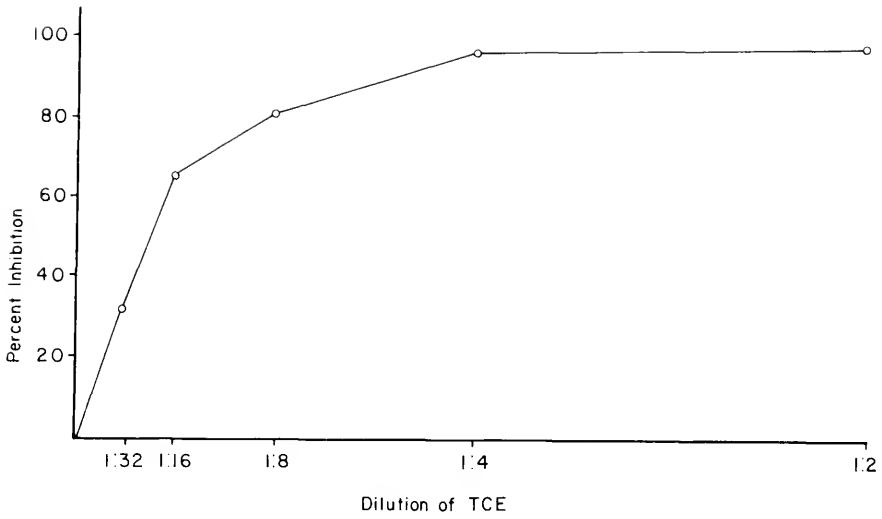

100
80
60
40
20
Percent Inhibition
1:32
1:16
1:8
1:4
1:2
Dilution of TCE

Figure 4. Inhibition of rabbit complement activity by Ehrlich ascites tumor cell extracts.

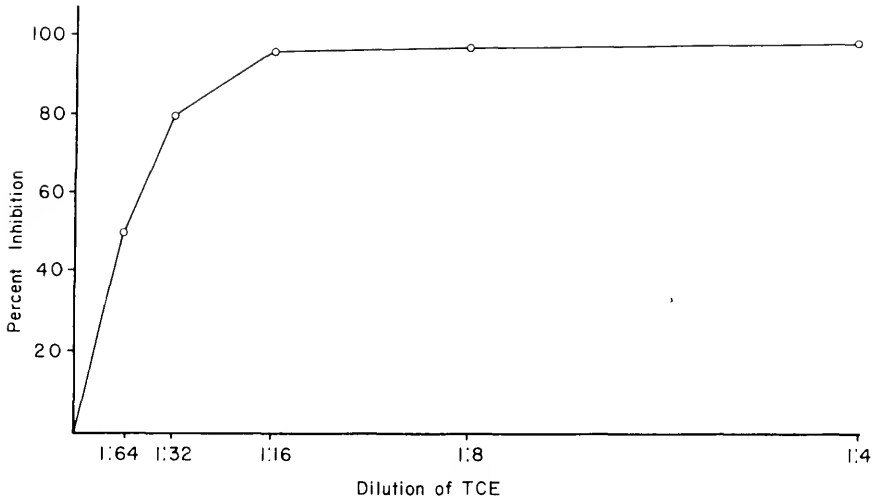

100
80
60
40
20
Percent Inhibition
1:64
1:32
1:16
1:8
1:4
Dilution of TCE

Figure 5. Inhibition of mouse complement activity by Ehrlich ascites tumor cell extracts.

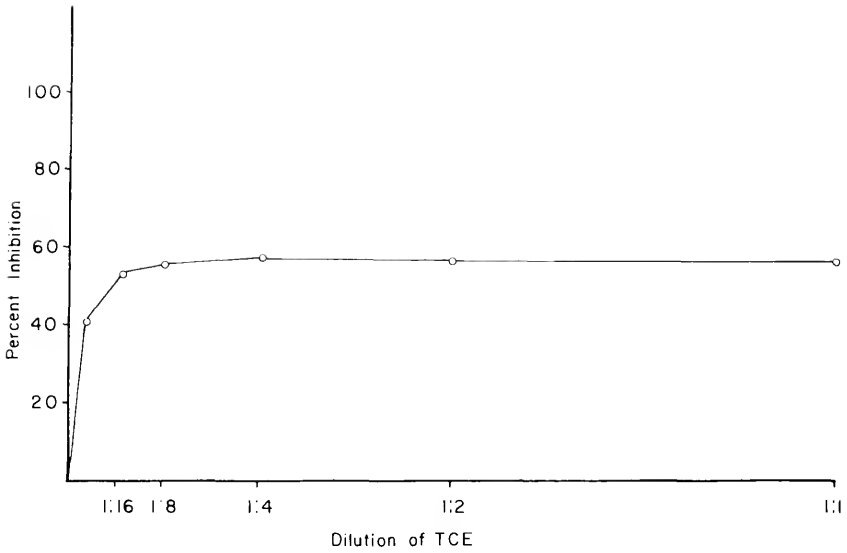
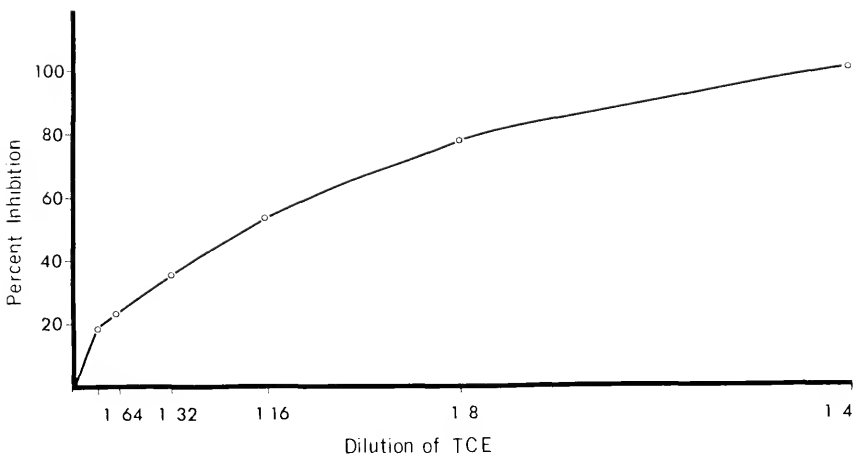

100
80
60
40
20
Percent Inhibition
1:16
1:8
1:4
1:2
1:1
Dilution of TCE

Figure 6. Inhibition of human complement activity by Ehrlich ascites tumor cell extract.


Percent Inhibition
100
80
60
40
20
1 64
1 32
1 16
1 8
1 4
Dilution of TCE

fixation resulting from natural antibodies in the complement source reacting with tumor antigens in the tumor cell extract even though the complement was routinely adsorbed with intact EATC. A 1:2 dilution of adsorbed guinea pig complement was incubated with a 1:5 dilution of EATC extract for 15 min at 30°C. The complement was then diluted to 1:300 and titrated as by Mayer (92). The inhibitory activity of the tumor extract used showed 100 percent inhibition at 1:5 dilution and no inhibition at a 1:256 dilution. Figure 7 shows that there is no apparent complement fixation by EATC extracts under the described conditions. Figure 7 also shows that the PBS extract does not seem to have any fluid phase effect on whole complement activity in guinea pig serum.

Inhibition of hemolytic antibody titration by tumor cell extracts. Extracts from EATC were incubated with antiserum against sheep erythrocytes to see if anti-sheep erythrocyte antibodies reacted with EATC extracts. The inhibition of whole complement could be a result of the antibodies reacting with the tumor extract and interfering in some way with lysis of sheep erythrocyte target cells. EATC tumor extracts and PBS were each incubated with separate samples of anti-sheep erythrocyte hemolysin and the antiserum was titered as described by Mayer (97). The titration of the antiserum as shown in Figure 8 shows that extracts from EATC did not affect the titration of antibodies to sheep red cell antigens. If the

Figure 7. Titration of adsorbed guinea pig complement after treatment with EATC extracts. The closed circles (●—●) show the titration of guinea pig serum after incubation with PBS and the open circles (○—○) denote the titration of guinea pig serum after incubation with PBS extract of EATC.

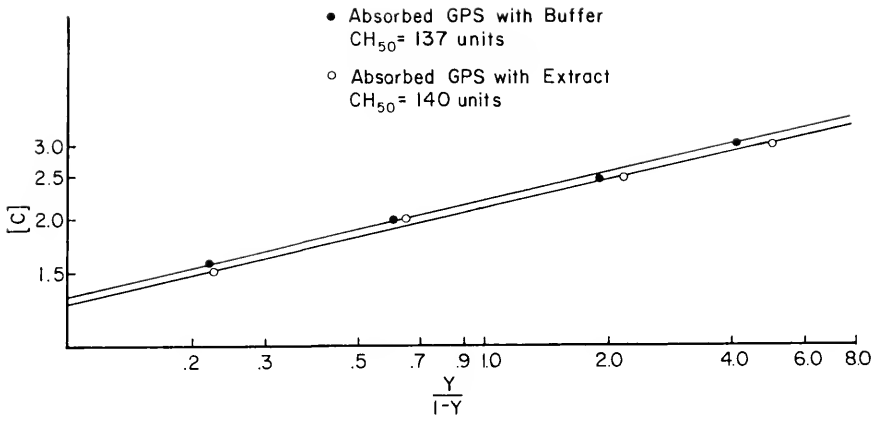
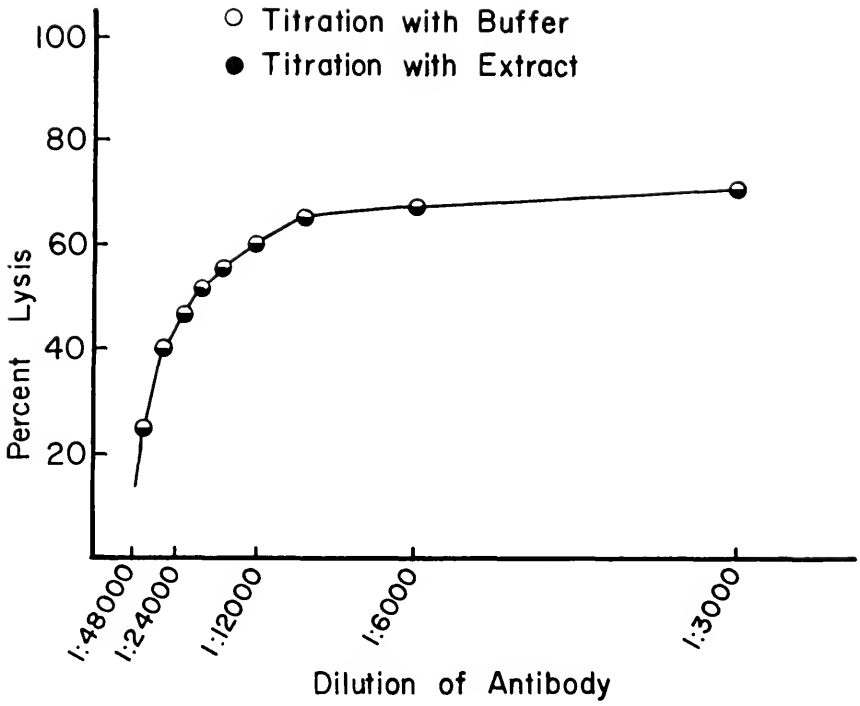

• Absorbed GPS with Buffer
CH50= 137 units
○ Absorbed GPS with Extract
CH50= 140 units
[C]
3.0
2.5
2.0
1.5
.2
.3
.5
.7
.9
1.0
2.0
4.0
6.0
8.0
Y/1-Y

Figure 8. Effect of EATC extracts on hemolytic antibody titration. Incubation of tumor cell extracts (●—●) with antiserum to sheep erythrocytes shows no apparent effect on the titration of the antiserum. Titration of control treated antiserum is given by the open circles (○—○).

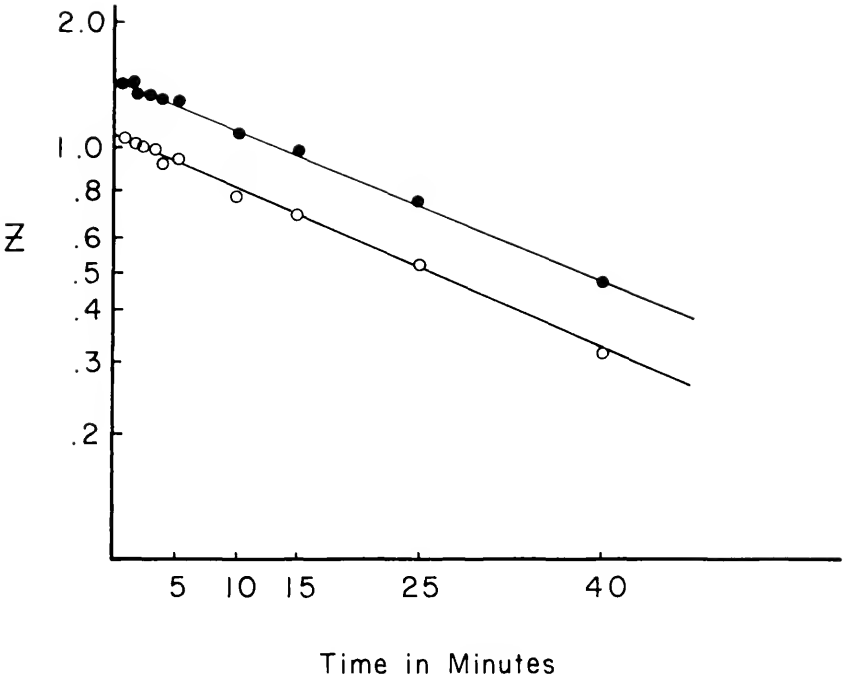

○ Titration with Buffer
● Titration with Extract
100
80
60
40
20
Percent Lysis
1:48000
1:24000
1:12000
1:6000
1:3000
Dilution of Antibody

tumor cell extract did cross react with sheep erythrocyte antigens the titer of the antiserum would be lower in the presence of the extract.

Decay of SAC$\overline{1}\overline{42}$ in the presence of EATC extracts. Extracts from human erythrocytes have been shown to accelerate the decay of the sheep erythrocyte, rabbit antibody, complement component intermediate, EAC$\overline{1}\overline{42}$ (88). EATC extracts were incubated with EAC$\overline{1}\overline{42}$ to see what effect this material had on the decay rate of the intermediate. One volume each of tumor cell extract or PBS was mixed with an equal volume of the intermediate EAC$\overline{1}\overline{42}$ at 0°C. The mixtures were placed at 30°C and the rates of decay were followed. Figure 9 shows that EATC extracts did not accelerate the decay of the intermediate EAC$\overline{1}\overline{42}$ since the two EAC$\overline{1}\overline{42}$ decay curves are approximately parallel. The extract does impair the ability of the cells in this intermediate state to undergo lysis in the presence of C-EDTA (source of C3 through C9) since there was less lysis of EAC$\overline{1}\overline{42}$ in the presence of EATC extract.

Effect of tumor extract on the stability of SAC$\bar{1}$4. Extracts from rabbit and guinea pig stromata have been shown to cause a time dependent inactivation of EAC$\bar{1}$4 at 30°C (89). The crude PBS extract of EATC was mixed with an equal volume of EAC$\bar{1}$4 (1 X 10^8/ml) and placed at 30°C. The control consisted of incubating PBS with EAC$\bar{1}$4. Samples were taken at timed intervals, the cells were washed twice in DGVB^{++} and assayed for SAC$\bar{1}$4 (98). As can

Figure 9. Decay of EAC$\overline{1}\overline{4}\overline{2}$ at 30° in the presence of tumor cell extracts. The upper curve (●—●) shows the decay of the intermediate treated with PBS. The lower curve (○—○) shows the decay of the intermediate treated with a PBS extract of Ehrlich ascites tumor cells. Z = average number of SAC$\overline{1}\overline{4}\overline{2}$ per cell.


2.0
1.0
.8
.6
.5
.4
.3
.2
Z
5
10
15
25
40
Time in Minutes

be seen in Figure 10, there is no effect of crude tumor cell extract on the stability of the EAC$\overline{1}$4 intermediate. It is evident, however, that EAC$\overline{1}$4 treated with the tumor extract undergo less lysis as compared to controls. The rate of inactivation must be rapid since the inactivation was complete within two min at 30°C.

Site of hemolytic inhibition by EATC extracts. In order to find out what steps in the complement sequence were affected by the crude salt extract of Ehrlich ascites tumor cells, sheep E, EA, and all of the cellular intermediates in the complement sequence were incubated with the tumor extracts. Each intermediate to be tested was adjusted to 1 X 10^8/ml in $DGVB^{++}$, mixed with an equal volume of the PBS extract from EATC and incubated for 15 min at 30°C. After incubation, the cells were washed twice and assayed for reactivity as compared to PBS treated cells. The results shown in Table 1 indicate that the early steps in the complement sequence were inhibited by EATC extracts. The inhibitory material showed no inactivation of sheep erythrocytes, but did show marked inactivation of EA, EAC$\overline{1}$, EAC4, and EAC1$\overline{4}$. The intermediate EAC$\overline{1\,4\,2}$ was inhibited to a lesser extent and once C2 was on the cells, the EATC extracts had no effect on the later reacting components.

Effect of tumor cell extract on the uptake of C1 by EA. Treatment of EA with tumor cell extract led to an impairment of the ability of complement to lyse the cells. Therefore, the ability of extract treated and untreated EA

Figure 10. Effect of EATC extract on the stability of the intermediate $EAC\bar{1}4$. The upper curve (●—●) shows the stability of $EAC\bar{1}4$ at 30°C in the presence of PBS. The lower curve (○—○) shows the stability of $EAC\bar{1}4$ in the presence of a PBS extract of EATC. Z = average number of $SAC\bar{1}4$ per cell.

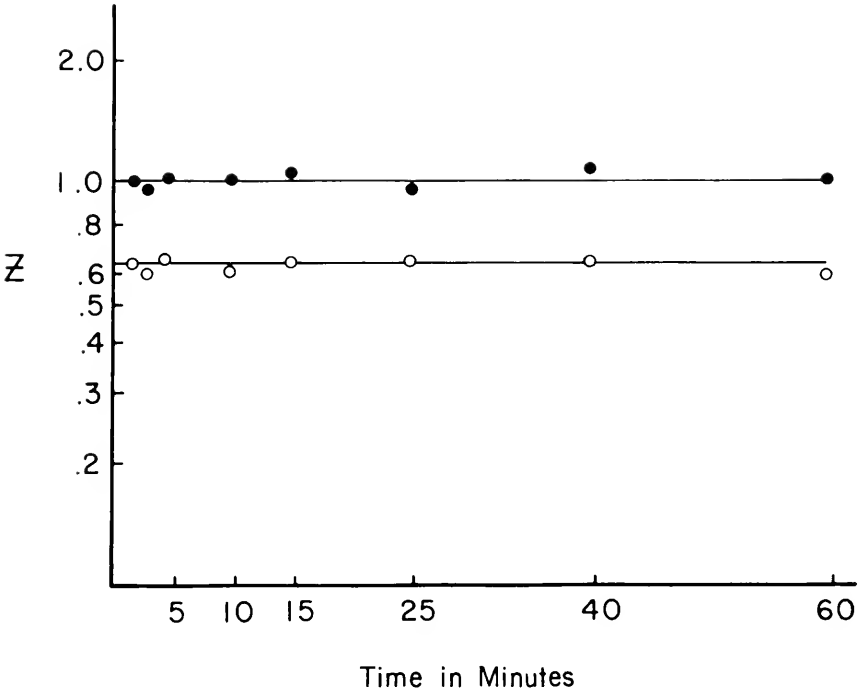

2.0
1.0
.8
.6
.5
.4
.3
.2
Z
5
10
15
25
40
60
Time in Minutes

TABLE 1

Effect of EATC Extract on the Hemolytic Susceptibility of Sheep E, EA, $EAC\overline{1}$, $EAC\overline{1}4$, EAC4, $EAC\overline{1}\overline{42}$, $EAC\overline{1-3}$, $EAC\overline{1-5}$, $EAC\overline{1-6}$, $EAC\overline{1-7}$ and $EAC\overline{1-9}$

Intermediate	Percent Hemolysis		Percent Inhibition
	Treated	Control	
E	53	55	3
EA	25	73	65
$EAC\overline{1}$	30	77	61
EAC4	27	61	55
$EAC\overline{1}4$	42	64	34
$EAC\overline{1}\overline{42}$	59	73	19
$EAC\overline{1-3}$	50	52	4
$EAC\overline{1-5}$	62	62	0
$EAC\overline{1-6}$	73	75	2
$EAC\overline{1-7}$	74	76	2
$EAC\overline{1-9}$	54	55	1

to fix C1 was assayed using the method of C1 fixation and transfer. EA (1 X 10^8/ml) were treated with tumor cell extract or with PBS; the cells were washed and resuspended to a cell concentration of 1 X 10^8/ml. The cells were then reacted with guinea pig C1 and the amount of C1 fixed in each case was measured in the C1 transfer test. Table 2 shows that EA treated with tumor cell extract (EA_T) fixed more C1 than did PBS treated EA (EA_C). The C1 fixed to the tumor cell extract treated EA was not inactivated since active C1 was being measured with the C1 fixation and transfer procedure. Although tumor cell extract treated EA were capable of fixing more C1 than control cells, the cells were inhibited from lysing with guinea pig complement as shown in Table 1.

Consumption of C4 by tumor cell extract treated $EAC\bar{1}$. $EAC\bar{1}$ were treated with the tumor cell extract to determine if the extract had any effect on the reactivity of C1 on $EAC\bar{1}$ cells. This experiment was performed to investigate the effects of tumor cell extract on the ability of $EAC\bar{1}$ to consume C4. $EAC\bar{1}$ were generated and then treated with tumor cell extract or PBS. $EAC\bar{1}$ treated with extract are designated $EAC\bar{1}_T$. $EAC\bar{1}$ treated with PBS are designated $EAC\bar{1}_C$. The treated $EAC\bar{1}$ cells were then used to measure consumption of C4. $EAC\bar{1}$ were reacted with C4; the mixtures centrifuged, and the residual C4 activity present in the supernatent fluids was titrated. Figure 11 shows that $EAC\bar{1}$ treated with tumor cell extract ($EAC\bar{1}_T$) consumed less C4 than did $EAC\bar{1}_C$ since more C4 was titratable in the supernatent after treatment.

TABLE 2

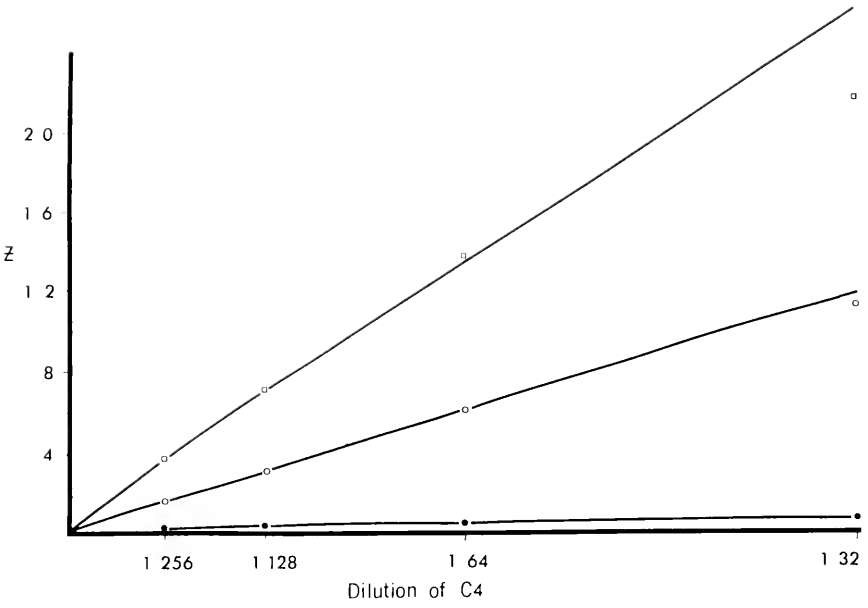
Comparison of the Relative Numbers of Effective $C\bar{1}$ Molecules Fixed by EA Treated with EATC Extracts and by Untreated EA

Sample	Experiment Number	Effective Number of $C\bar{1}$ Molecules Fixed/Cell	Standard Deviation
EA_T [a]	1	245	± 4
	2	173	±35
	3	227	±17
EA_C [b]	1	144	± 6
	2	133	±13
	3	148	±18

a) EA treated with tumor cell extract at 30°C for 15 min before addition of Cl.

b) EA treated with PBS at 30°C for 15 min before addition of Cl.

Figure 11. Titration of residual C4 activity after treatment with $EACl_T$ and $EACl_C$ cells. The upper curve represented by the open squares (■—■) represents the titration of untreated C4. The middle curve (O—O) shows the titration of residual C4 after treatment with $EACl_T$. The lower curve represents the titration of residual C4 after treatment with PBS treated cells ($EACl_C$). Z = average number of $SAC\bar{l}4$ per cell.


2 0
1 6
Ẑ
1 2
8
4
1 256
1 128
1 64
1 32
Dilution of C4

Effect of EATC extracts on the generation of $SAC\overline{1}\overline{42}$. Tumor cell extracts were reacted with cells in the intermediate state of $EAC\overline{1}4$ to determine if the extract was capable of affecting the generation of $SAC\overline{1}\overline{42}$ from $SAC\overline{1}4$. Equal volumes of $EAC\overline{1}4$ (1 X 10^8/ml) were incubated with equal volumes of either EATC extracts or PBS for 15 min at 30°C. The cells were washed and resuspended to 1 X 10^8/ml and the Tmax was determined. The results given in Figure 12 show that $EAC\overline{1}4$ which had been treated with tumor cell extract had a longer Tmax than did a buffer treated control, thus indicating that the treatment caused a decrease in the available number of $SAC\overline{1}4$ on the cells (98).

Effect of tumor cell extracts on individual complement components. The tumor cell extract was incubated with C1 and C2 to determine if the extract was capable of inactivaing fluid phase C1 and C2. Equal volumes of C1 at a 1:5 dilution were incubated with EATC extracts or PBS for 15 min at 30°C and the C1 was titrated in the standard assay system (93). Figure 13 shows that treatment of C1 with tumor extracts had no effect on titer of the component using an incubation time and temperature equivalent to those used when EATC extract was incubated with $EAC\overline{1}$. C2 at a dilution of 1:4 was incubated with tumor cell extracts in the same manner as was C1 and the C2 was titered as described. Figure 14 shows that the titration curves of C2 treated with PBS or with the tumor cell extract were essentially the same. No evidence was found for fluid phase inactivation of C2 using

Figure 12. Effect of EATC extracts on the generation of SAC$\overline{142}$. The curve denoted by the closed circles (●—●) represents SAC$\overline{142}$ generation on EAC$\overline{14}$ cells treated with PBS. The curve denoted by the open circles (○—○) represents the generation of SAC$\overline{142}$ on EAC$\overline{14}$ treated with tumor cell extract. Z = average number of SAC$\overline{142}$ per cell.

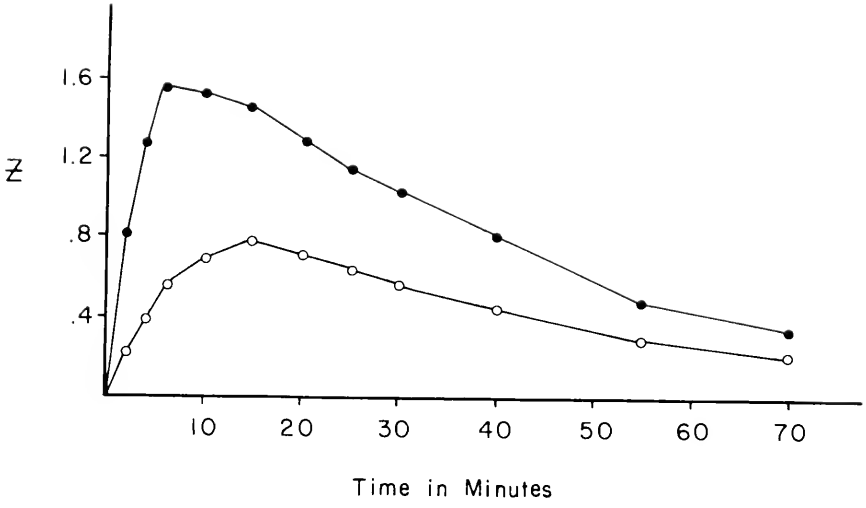

1.6
1.2
.8
.4
10
20
30
40
50
60
70
Time in Minutes

Figure 13. Titration of C1 treated with EATC extracts (●—●) and with PBS (○—○). $\bar{Z}$ = average number of SAC$\bar{1}$ per cell.

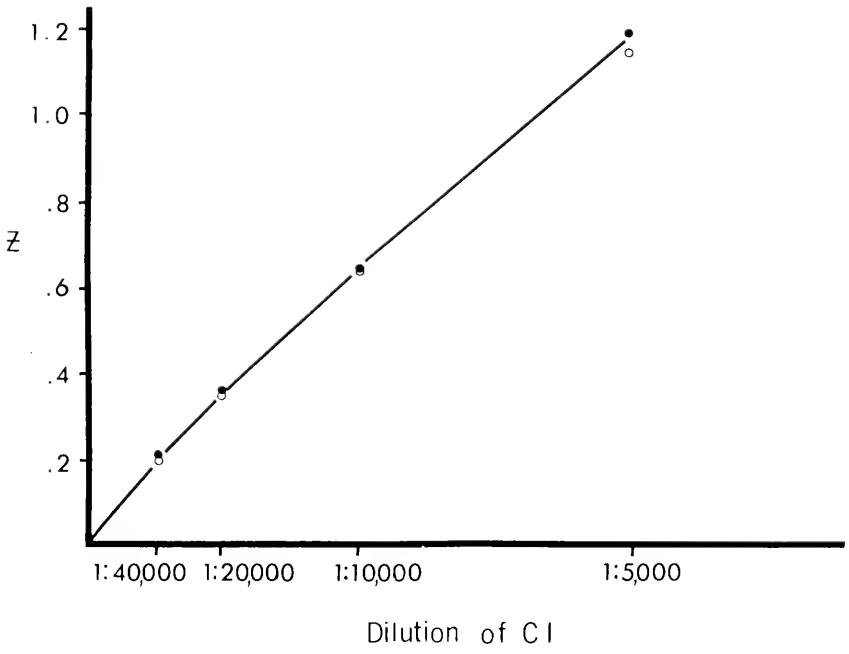
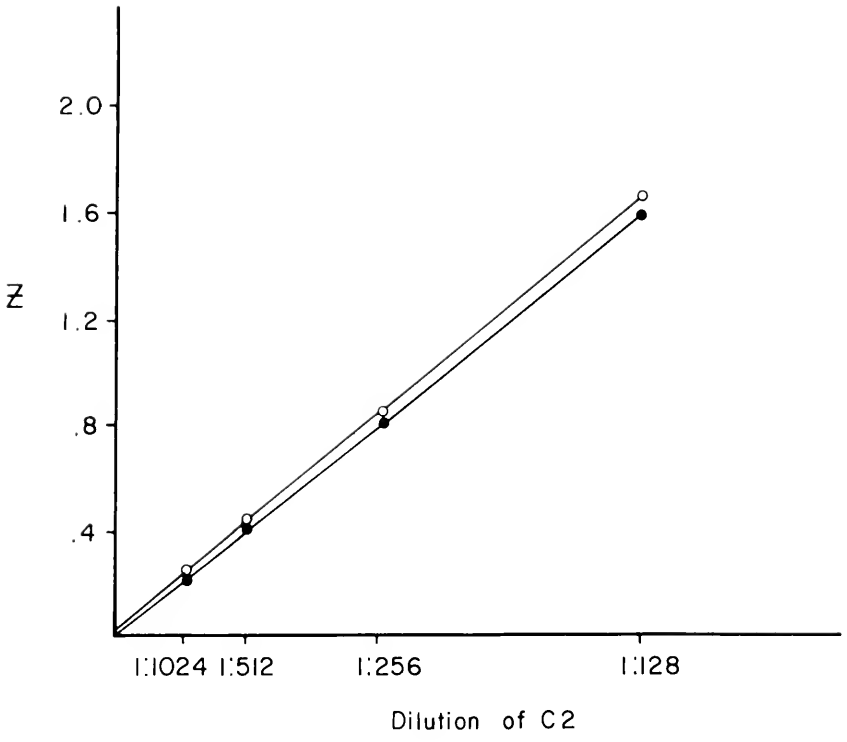

1.2
1.0
.8
Z
.6
.4
.2
1:40,000
1:20,000
1:10,000
1:5,000
Dilution of C I

Figure 14. Titration of C2 treated with EATC extracts (●—●) and with PBS (○—○). Z = average number of SAC$\overline{1}\overline{4}\overline{2}$ per cell.

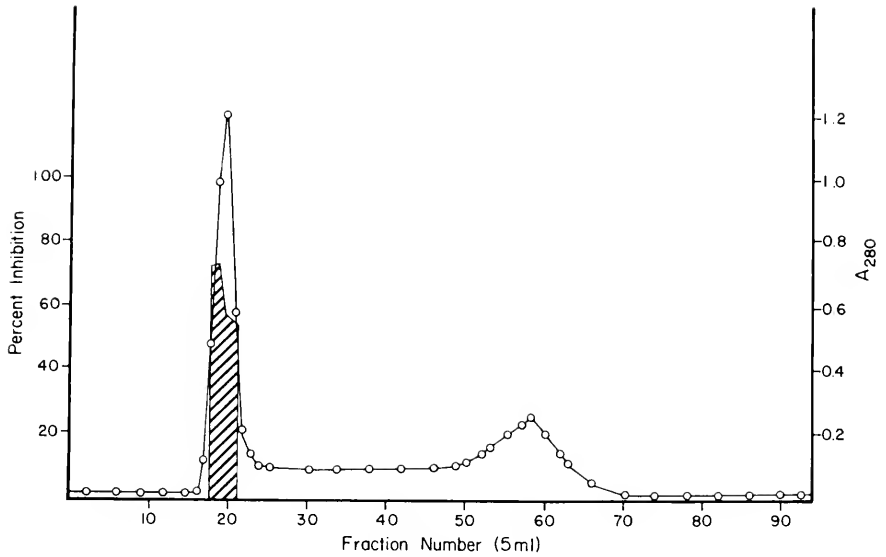

2.0
1.6
1.2
.8
.4
Z
1:1024
1:512
1:256
1:128
Dilution of C2

incubation conditions which were the same as when EAC$\overline{142}$ were incubated with EATC extract.

Preliminary purification attempts. Since the tumor cell extract could inhibit at several points in the early sequence of complement activation, it was difficult to come up with a model that could explain all of the phenomena that were observed. Therefore, it was necessary to attempt to purify the inhibitory substance or substances in the tumor cell extract.

A combination of gel filtration, ammonium sulfate fractionation and DEAE chromatography was utilized initially in an attempt to purify the active inhibitory material in the PBS extracts of EATC. Crude tumor cell extract was first applied to a G-200 Sephadex column and the inhibitory activity eluted immediately after the void volume (Figure 15). Since Sephadex gave no additional purification, the next approach to the partial purification of the tumor cell extract was fractional ammonium sulfate precipitation. The inhibitory activity precipitated at a concentration of 20 percent saturation with ammonium sulfate and less than 10 percent of the inhibitory activity was found in the other ammonium sulfate fractions. The 20 percent ammonium sulfate precipitate was redissolved and dialyzed overnight versus PBS. The volume of the dialyzed fraction (50 ml) represented one-half of the original starting volume. The crude tumor cell extract was capable of causing 50 percent inhibition at a 1:512 dilution. The redissolved ammonium sulfate

Figure 15. Gel filtration of PBS extract from EATC on G-200 Sephadex. The column was equilibrated with PBS. Five milliliter volumes of the crude tumor cell extract were applied. The fractions were collected and the absorbancy at 280nm was determined, as shown by the open circles (O—O). The fractions capable of inhibiting immune hemolysis are shown by the cross-hatched area. Void volume = 100ml.


Percent Inhibition
100
80
60
40
20
1.2
1.0
0.8
0.6
0.4
0.2
A_{280}
10
20
30
40
50
60
70
80
90
Fraction Number (5ml)

precipitate caused 50 percent inhibition at a 1:400 dilution. One half of the redissolved precipitate (25 ml) was adjusted to an ionic strength of 0.3, pH 7.5, and applied to a DEAE column that was equilibrated at an ionic strength of 0.3, pH 7.5. After application, the column was washed with the equilibrating buffer and a linear sodium chloride gradient was initiated. The inhibitory material bound to the DEAE column at an ionic strength of 0.3 and it began to elute at an ionic strength of 0.4. The elution peak was at an ionic strength of 0.55. The absorbancy of each fraction was determined at a wavelength of 280nm. The fractions were then adjusted to an ionic strength of 0.15 and assayed for inhibitory activity as shown in Figure 16. The input material caused 50 percent inhibition of complement mediated lysis of EA at a 1:400 dilution.

The entire inhibitory peak obtained after DEAE chromatography was pooled (fractions 37 through 70), precipitated with saturated ammonium sulfate. The precipitate was redissolved in 8 ml of PBS, dialyzed and 2 ml was applied to a Bio-Gel A-1.5m column. The column was eluted with PBS and the optical density at 280nm of each fraction was determined. The inhibitory activity was retained by the gel and all of the inhibitory activity eluted in a single peak which coincided with the optical density at 280nm (Figure 17). The input material caused 50 percent inhibition of EA lysis at a dilution of 1:16. The fractions containing inhibitory activity were pooled(fractions

Figure 16. DEAE chromatography of the 20 percent ammonium sulfate precipitated inhibitory material from the EATC extracts. The inhibitory activity is given by the cross-hatched area. The open circles (O—O) show the optical density at 280nm, and the dashed line shows the sodium chloride gradient.

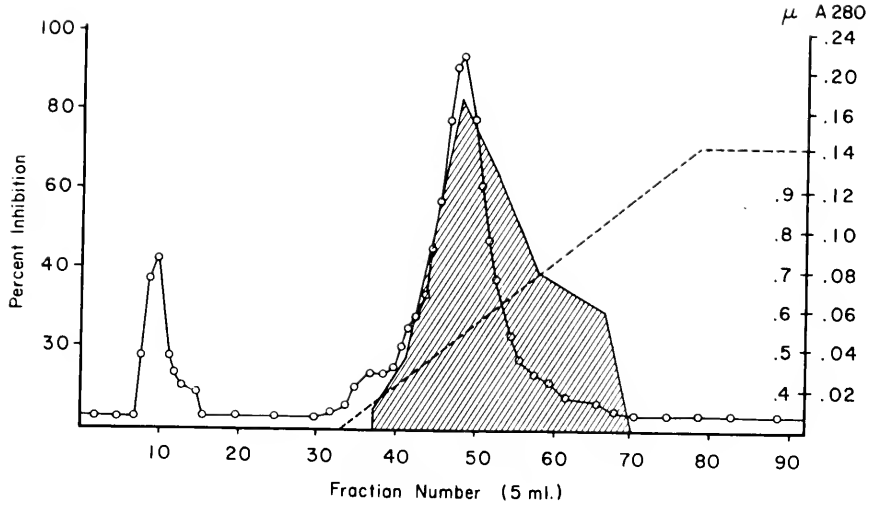
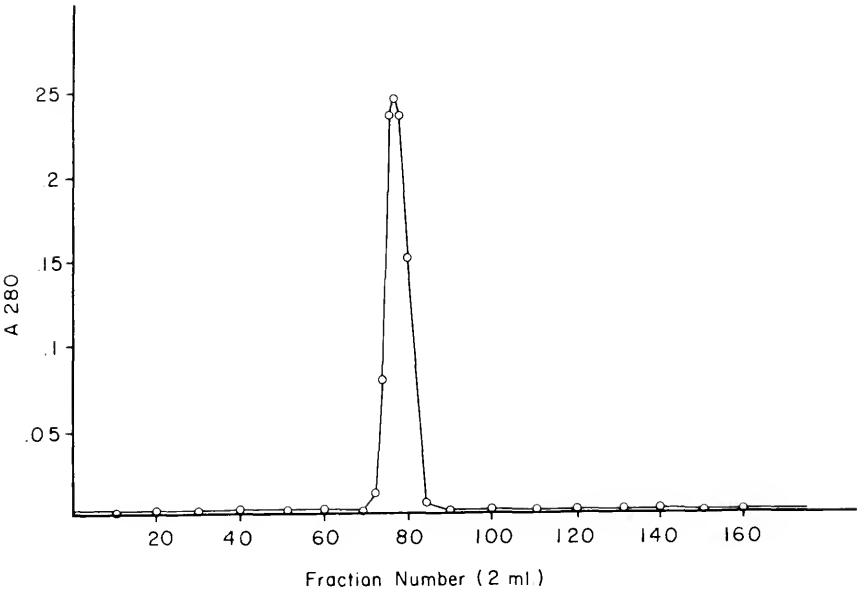

μ A280
.24
.20
.16
.14
.12
.10
.08
.06
.04
.02
.9
.8
.7
.6
.5
.4
100
80
60
40
30
Percent Inhibition
10
20
30
40
50
60
70
80
90
Fraction Number (5 ml.)

Figure 17. Gel filtration of DEAE purified tumor cell extract on Bio-Gel A-1.5m. The optical density at 280nm is given by the open circles (O—O). The inhibitory activity coincided with the peak of A280 absorbing material. After concentration, the purified extract was capable of causing 50 percent inhibition at a 1:2 dilution. Void volume = 60 ml.

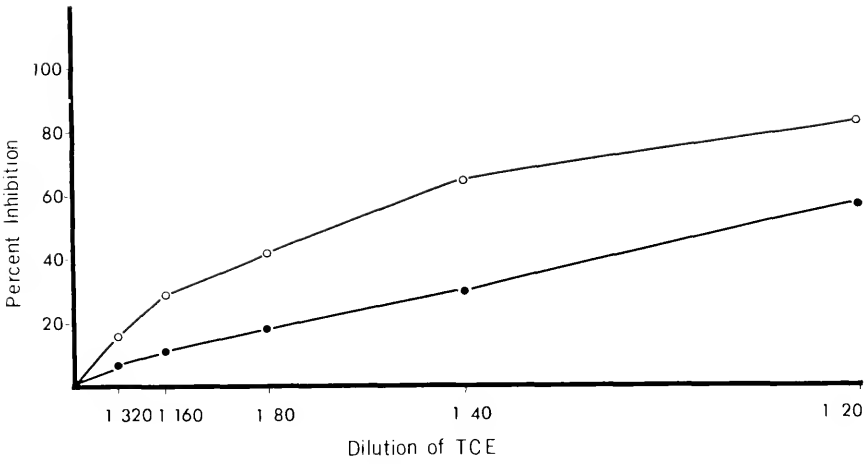

25
2
.15
.1
.05
A 280
20
40
60
80
100
120
140
160
Fraction Number (2 ml)

72 through 80) and concentrated 8-fold. After concentration, a 1:2 dilution of this purified material was capable of causing 50 percent inhibition of complement mediated lysis of EA. Although these methods could achieve purification and recover inhibitory material, the yield of inhibitory material was less than one percent and therefore the procedure was generally unsatisfactory.

Sensitivity of tumor cell extracts to enzyme treatment. The tumor cell extracts were subjected to enzyme treatment in order to determine what class of compound was responsible for the inhibitory activity. Determining the class of compounds responsible for the activity would also provide an insight as to the type of purification scheme that should be developed. The PBS extract from EATC was not sensitive to the action of deoxyribonuclease since there was no loss of activity after treatment with the enzyme. Trypsin treatment of the tumor cell extract caused only a partial reduction in the inhibitory activity as shown in Figure 18. The inactivation of the extract could not be increased even when the concentration of trypsin was increased 10-fold. The control containing trypsin plus trypsin inhibitor showed no complement inhibitory activity.

The sensitivity of the EATC extract to protease was tested using an enzyme bound to agarose by the azyl azide procedure (103). Crude tumor cell extract was incubated with the enzyme on the column and eluted with VB^{++}. The inhibitory activity of the extract after treatment was

Figure 18. Inactivation of EATC extract by trypsin treatment. Tumor cell extracts were incubated with trypsin and with buffer. The mixtures were then titrated for inhibitory activity. The inhibitory curve for tumor cell extract treated with buffer is given by the open circles (O—O). The inhibition curve of the tumor cell extract after treatment with trypsin is given by the closed circles (●—●).


100
80
60
40
20
Percent Inhibition
1 320
1 160
1 80
1 40
1 20
Dilution of TCE

compared with an extract that was diluted 1:2 with VB^{++} to correct for the dilution of the extract on the column. The buffer control consisted of incubating VB^{++} with the enzyme instead of the tumor extract. Figure 19 shows that there is only a small amount of inactivation of the tumor cell extract by the insolubilized protease. Increasing the time of enzyme treatment did not increase the amount of inhibitor inactivated. The differences in the susceptibility of the extract to trypsin and protease treatment could reflect the differences in the specific activities of the enzymes used.

The tumor cell extract was also tested for its susceptibility to the action of ribonuclease A. Figure 20 shows that the treatment of tumor cell extract with ribonuclease inactivated all of the inhibitory activity associated with the tumor cell extract. Interaction of ribonuclease with EA and complement did not show any effects by the enzyme on complement mediated lysis of EA. These findings indicated that the active inhibitory material contained RNA as a functional entity.

Streptomycin sulfate fractionation. Streptomycin sulfate has been used to precipitate nucleic acids from enzyme preparations (100). Therefore, a PBS extract from EATC was incubated with streptomycin sulfate to determine if the inhibitory activity of the tumor extract could be removed by this treatment. EATC extract was incubated with streptomycin sulfate for 10 min at 25°C and then for

Figure 19. Inactivation of EATC extract by protease. Inhibition of complement mediated lysis by untreated tumor cell extract is given by the closed circles (●—●). The curve denoted by the open circles (○—○) shows the inhibitory activity after treatment with protease. The closed squares (■—■) show the inhibition caused by the buffer control.

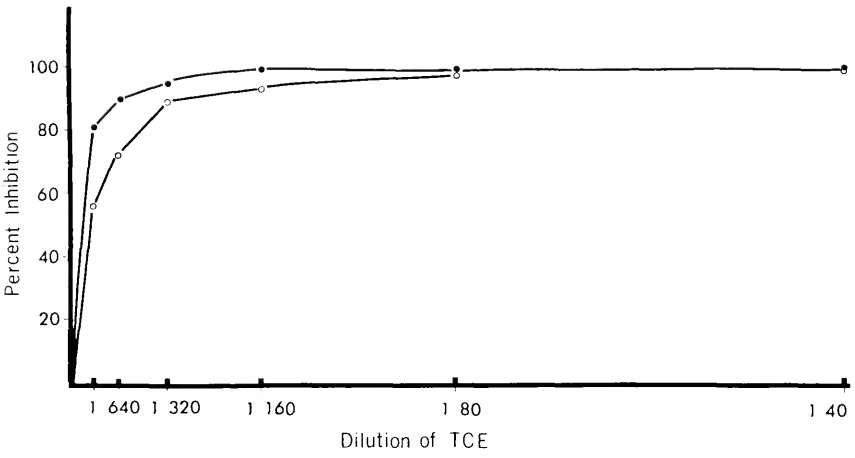
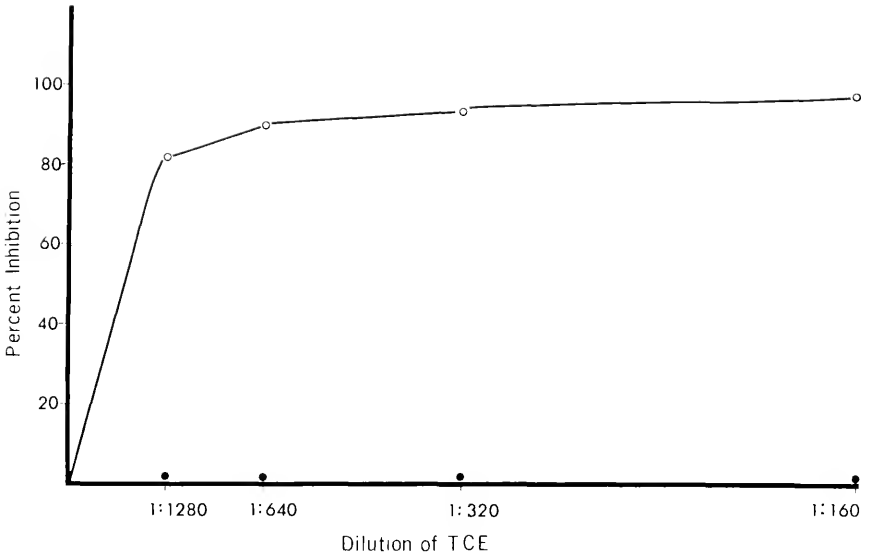

100
80
60
40
20
Percent Inhibition
1 640
1 320
1 160
1 80
1 40
Dilution of TCE

Figure 20. Inactivation of EATC extract by ribonuclease A. Tumor cell extracts treated with ribonuclease and with buffer were titrated for inhibitory activity in the immune hemolysis inhibition test. The curve represented by the open circles (O—O) shows the inhibitory activity of the extract with buffer. The closed circles (●—●) show the inhibitory activity of the extract treated with ribonuclease.


100
80
60
40
20
Percent Inhibition
1:1280
1:640
1:320
1:160
Dilution of TCE

18 hours at 4°C. An insoluble precipitate formed and was removed by centrifugation at 10,000G for 30 min. The supernate was dialyzed overnight versus PBS and titrated for inhibitory activity. Figure 21 shows that the supernate remaining after streptomycin treatment did not possess any inhibitory activity as compared to untreated EATC extracts when tested in the immune hemolysis inhibition test, thus providing additional evidence that the inhibitor was nucleic acid.

Partial purification of a complement inhibitory material from EATC using DEAE chromatography and phenol extraction. Since the original attempt to purify the inhibitory component from the EATC extract was not fruitful, another attempt was made to partially purify the inhibitory substance. A combination of DEAE chromatography and phenol extraction was used since the original DEAE chromatography gave good separation of inhibitory material from inactive contaminants and phenol extraction procedures are used to purify RNA (101).

A PBS extract from EATC capable of causing 50 percent inhibition of complement mediated lysis of EA at a 1:2000 dilution was adjusted to an ionic strength of 0.3, pH 7.5, and applied to DEAE which was equilibrated at the same pH and ionic strength. The column was washed with buffer (ionic strength = 0.3, pH 7.5) and a linear salt gradient was initiated. Fractions were collected and the absorbancy of each fraction was determined at wave -

Figure 21. Removal or inactivation of EATC extract inhibitory material with streptomycin sulfate. The curve depicted by the open circles (○—○) shows the inhibition caused by untreated tumor extracts. The closed circles (●—●) represent the inhibition of complement mediated lysis after the extract was treated with streptomycin sulfate.

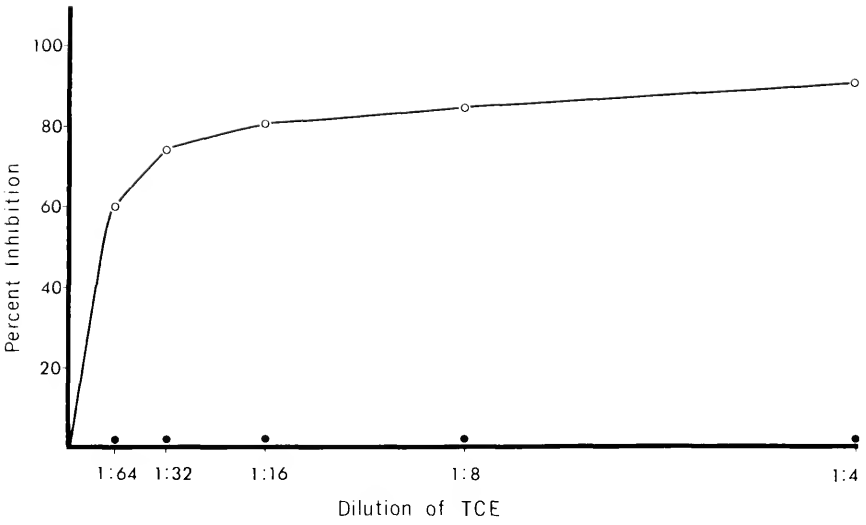
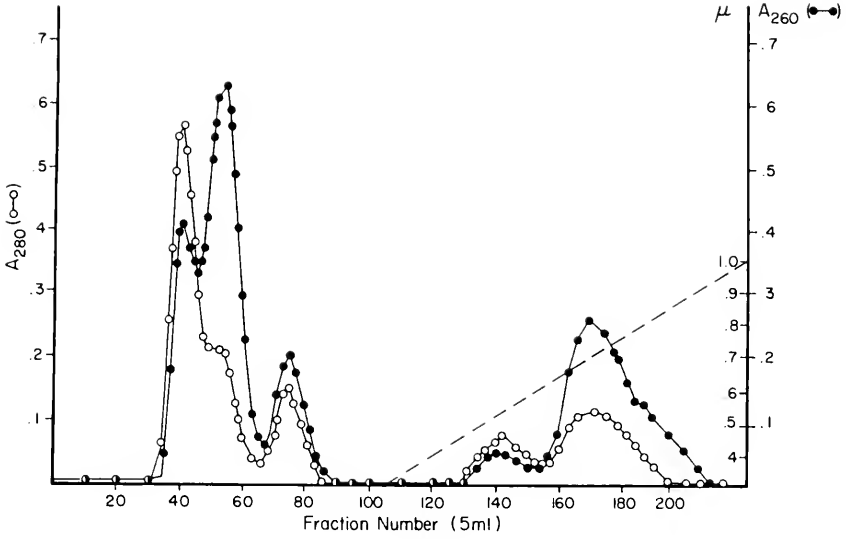

100
80
60
40
20
Percent Inhibition
1:64
1:32
1:16
1:8
1:4
Dilution of TCE

Figure 22. DEAE chromatography of PBS extract from EATC. The open circles (O—O) represent the optical density at 280nm and the closed circles (●—●) represent the absorbancy at 260nm. The dashed line shows the sodium chloride gradient. The input was 10.0 ml of crude extract which had an optical density at 280nm of 6.10 and at 260nm of 10.44. The crude extract was capable of causing 50 percent inhibition of EA lysis at a dilution of 1:2000.


μ
A260 (●—●)
A280 (○—○)
.7
.6
.5
.4
.3
.2
.1
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
20
40
60
80
100
120
140
160
180
200
Fraction Number (5ml)

lengths of 280nm and 260nm. The elution profile presented in Figure 22 shows that a number of peaks resolved which varied in their relative absorbance at the two wavelengths. The fractions were then adjusted to an ionic strength of 0.15 with distilled water and each fraction was tested for its ability to inhibit complement mediated lysis. Only fractions 161 through 185 showed any inhibitory activity. The inhibitory activity bound to DEAE at an ionic strength of 0.3 and began eluting at an ionic strength of about 0.65 with the peak around 0.7. The fractions 35 through 45, 47 through 65, 70 through 83, 100 through 110, 130 through 155, 161 through 185, and 190 through 210 were pooled and extracted with aqueous phenol. Ethanol (95 percent) was added to the aqueous phase to precipitate nucleic acid. The precipitate was washed and redissolved in PBS at one-tenth the original fraction volume. The pooled concentrated fractions were assayed for inhibitory activity. Only the pool containing fractions 161 through 185 showed any inhibitory activity. The volume of the pooled fractions before phenol extraction was 125 ml and was capable of causing 50 percent inhibition of complement mediated lysis of EA at a 1:20 dilution. After phenol extraction, the concentrated fractions (161 through 185) were capable of causing 50 percent inhibition of complement mediated lysis of EA at 1:180 dilution. The DEAE purified phenol extracted material had an optical density at 280nm of .63 and an optical density at 260nm of 1.48. The ratio of the absorbance at wavelenghts

of 280nm/260nm was approximately 0.43. Purified yeast RNA obtained from Sigma Chemical Company gave a ratio of 280nm/260nm of 0.42. According to Warburg and Christian (104), a material with a ratio of 280nm/260nm of 1.75 contains essentially no nucleic acid and a material with a 280nm/260nm ratio of .49 contains 100 percent nucleic acid. The resultant yield of complement inhibitory activity from this purification procedure was approximately 15 percent. The specific activity of the purified extract (reciprical of the dilution causing 50 percent inhibition of immune hemolysis per optical density at 260nm) was approximately 150 and the specific activity of the crude extract was 190.

Extraction of inhibitory material from the 0.15M sodium chloride extract of EATC with phenol. Ribonuclease treatment of crude tumor cell extract eliminated all of the complement inhibitory activity of the extract. Phenol extraction of the inhibitory fractions from DEAE chromatography of the crude extract gave reasonable purification of the inhibitory material. Therefore, direct phenol extraction of the crude PBS extract from EATC was performed in an attempt to purify RNA in large enough quantities to investigate the mechanism of action of the partially purified material. A PBS extract was extracted with phenol and precipitated with 95 percent ethanol. The precipitate was redissolved in PBS, dialyzed against PBS and assayed for inhibitory activity according to the methods given in Materials and Methods. Using this procedure, 85 percent of

the inhibitory activity could be recovered. The ratio of absorbance at wavelenghts of 280nm/260nm of the crude extract was approximately 0.58. The ratio of 280nm/260nm of the phenol extracted material was 0.48. A material with a ratio of 280nm/260nm as low as 0.49 contains essentially all nucleic acid (104). Figure 23 shows the inhibition of complement mediated EA lysis caused by various dilutions of a phenol extract obtained from the PBS extracted inhibitory material. Since the phenol extraction procedure could recover 85 percent of the inhibitory material with good purification, the phenol extract was used to define the mechanism of inhibition of a partially purified tumor cell extract.

Heat sensitivity of tumor cell extracts. After obtaining the phenol extract, the PBS extracted material and the phenol extract were tested to see if the inhibitory components were stable to heating at 56°C. It was observed during the course of this investigation that various crude extracts from EATC varied in their inhibitory titers. This could be denaturation of the inhibitory compounds while the material was prepared and tested. The heat sensitivities of the crude extract and the phenol extract were compared to determine if they were stable to heating and if the variability of the inhibitory titers could be due to this phenomenon. A dilution of each extract capable of causing approximately 90 percent inhibition of EA lysis by guinea pig complement was placed in a 56°C water bath. At timed intervals, samples were taken and assayed for

Figure 23. Inhibition of immune hemolysis caused by a phenol extract of the crude, sodium chloride extract from EATC.

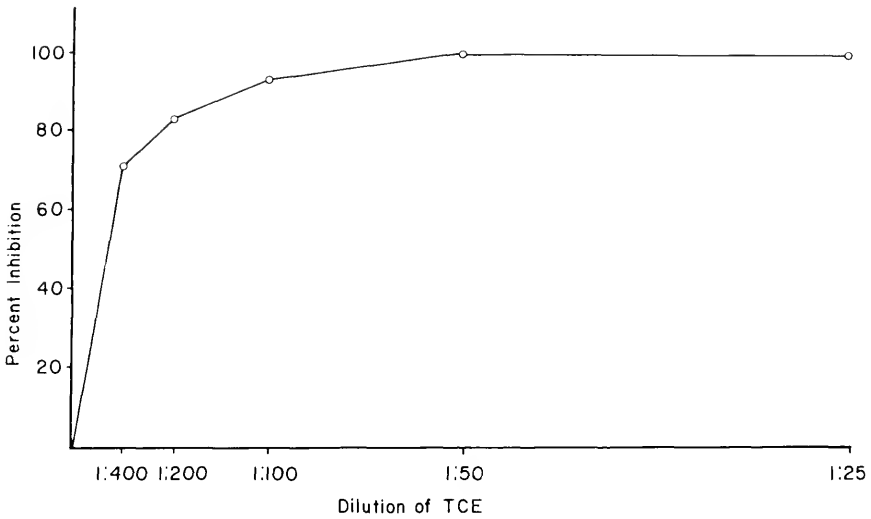
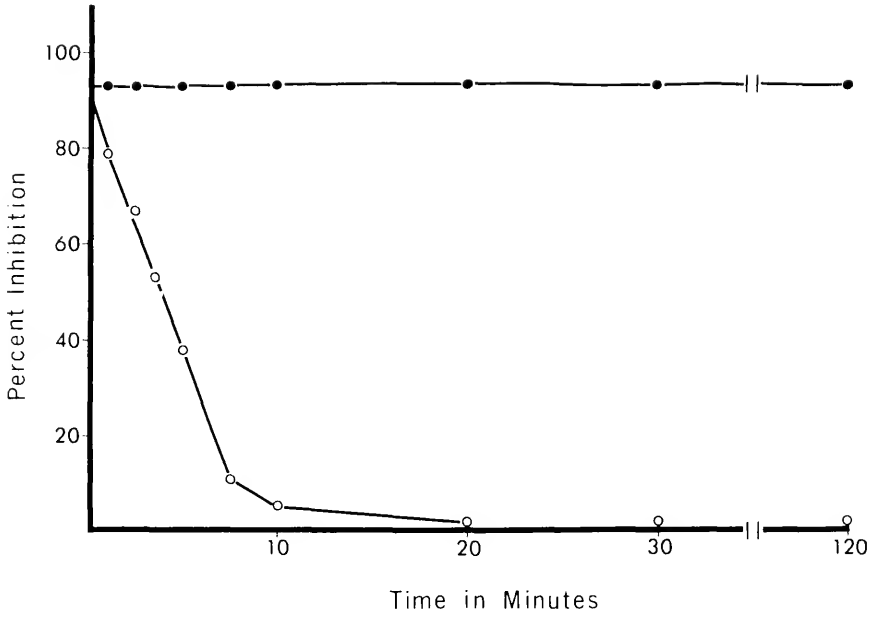

100
80
60
40
20
Percent Inhibition
1:400
1:200
1:100
1:50
1:25
Dilution of TCE

Figure 24. Heat stability of the crude tumor cell extract and the phenol extract at 56°C. The stability of the phenol extract is shown by the closed circles (●—●) and of the crude extract by the open circles (○—○).


100
80
60
40
20
Percent Inhibition
10
20
30
120
Time in Minutes

inhibitory activity. Figure 24 shows that the phenol extract is stable to heating at 56°C for at least 2 hours whereas the sodium chloride extract was inactivated completely by 20 min with a half-life of only 5 min at 56°C. The lability of the inhibitory substance itself could be due to the activation of a ribonuclease that may be present that could conceivably degrade the active material in the time tested.

Site of inhibition of immune hemolysis by phenol extracts. The inhibitory material obtained by extracting the PBS extract of EATC with aqueous phenol was tested on the early steps in the complement sequence to determine if the same intermediates were affected by the partially purified tumor cell extract as were inhibited by the crude material. Each intermediate to be tested was incubated with the phenol extract and with PBS. The cells were washed, restandardized and assayed for their ability to undergo lysis when the necessary reagents were added as described previously for the crude extract. As shown in Table 3, only the intermediates EA$C\bar{1}$ and EA$C\bar{1}4$ were affected by the phenol extracted inhibitory material as compared to the inhibition of EA, EA$C\bar{1}$, EAC4, EA$C\bar{1}4$, and EA$C\overline{1\,42}$ by the crude tumor cell extract.

Inability of C1 to transfer from phenol extract treated EA$C\bar{1}$. Since sheep erythrocytes coated with antibody were not inhibited or inactivated by phenol extract treatment, a C1 fixation and transfer test was not performed using EA as described for the crude extract. Instead, cells in the state EA$C\bar{1}$ were first generated and

TABLE 3

Effect of Phenol Extracts on the Hemolytic Susceptibitity of EA, EAC$\bar{1}$, EAC4, EAC$\bar{1}$4 and EAC$\overline{1}\overline{42}$

Intermediate	Percent Hemolysis		Percent Inhibition
	Treated	Control	
EA	74	77	4
EAC$\bar{1}$	4	71	95
EAC4	65	64	0
EAC$\bar{1}$4	8	72	89
EAC$\overline{1}\overline{42}$	88	87	0

then treated with phenol extract or with PBS, washed, resuspended to a cell concentration of 1 X 10^8/ml and the amount of C1 capable of transfer was measured in the C1 fixation test described in Materials and Methods. Table 4 shows that very few C1 molecules are capable of transferring from EAC$\bar{1}$ treated with the phenol extracted tumor cell material compared to PBS treated cells.

Inactivation of C1 in the fluid phase and on EAC$\bar{1}$ by phenol extracts. Phenol extracted tumor cell inhibitory material was incubated with C1 to compare the inactivation of this component by the crude extract and the partially purified phenol extracted material. Purified C1 was diluted 1:5 in $DGVB^{++}$ and one volume was incubated with an equal volume of the phenol extract at 30°C for 15 min. Another mixture was incubated at 30°C for 15 min, then for 18 hours at 4°C. After incubation, the mixtures were serially diluted and assayed for C1 activity (93). The relative numbers of C1 molecules present after treatment with the phenol extract were compared to PBS treated controls. Table 5 shows that there was no apparent inactivation of C1 by the phenol extract after treatment at 30°C for 15 min. Similarly, the crude tumor cell extract did not inactivate C1 under these conditions (Figure 13). Incubation of the extract with C1 for 18 hours at 4°C caused only partial inactivation of C1. In contrast, the phenol extract was capable of inactivating most of the C1 molecules of EAC$\bar{1}$ after a 15 min treatment at 30°C.

Inactivation of guinea pig complement components in whole sera by phenol extracts. The phenol extracted

TABLE 4

Comparison of the Relative Numbers of Effective $C\bar{l}$ Molecules Capable of Transfer from $EAC\bar{l}$ Treated with EATC Phenol Extracts

Sample	Experiment Number	Effective Number of $C\bar{l}$ Molecules Transferred/Cell
$EAC\bar{l}_T$ [a]	1	1.32
$EAC\bar{l}_T$	2	1.55
$EAC\bar{l}_C$ [b]	1	455
$EAC\bar{l}_C$	2	470

a) $EAC\bar{l}$ were generated and treated with phenol extract at 30°C for 15 min. The Cl capable of transfer was then titrated.

b) Control $EAC\bar{l}$ treated with PBS at 30°C for 15 min.

TABLE 5

Comparison of the Relative Numbers of Effective $C\bar{l}$ Molecules Inactivated in the Fluid Phase and on $EAC\bar{l}$ by EATC Phenol Extracts

Sample Treated	Time of Treatment	Number of Effective $C\bar{l}$ Molecules in System	Number of Effective $C\bar{l}$ Molecules Inactivated
$C\bar{l}$	15 min/30°C	1×10^{11}	0
$C\bar{l}$	18 hr/4°C	3.7×10^{10}	8.0×10^{9}
$EAC\bar{l}$	15 min/30°C	4.62×10^{10}	4.6×10^{10}

inhibitory substance was assayed for the ability to inactivate guinea pig complement components in whole sera. Table 6 shows that the extract was incapable of inactivating C1, C2, C4, or C3 after 15 min at 30°C. Sera that were treated with extract and incubated for 18 hours at 4°C were titrated for C1 and C2 activity. As shown in Table 6, only a partial inactivation of C1 or C2 was obtainable after this long treatment as compared to PBS treated control sera. The activity of C4 in serum after treatment with PBS or the phenol extracts for 18 hours at 4°C was too low to allow adequate titration of the component.

Reaction of Clq with partially purified extracts of EATC. The phenol extract was tested for its ability to react with purified human Clq since various polyanions such as RNA have been shown to react with Clq (17). Clq was incubated with the phenol extract and the capabity of the treated Clq to agglutinate gamma globulin coated latex particles was examined. Clq treated with phenol extract did not cause any reduction of the agglutinability of gamma globulin coated latex particles with Clq. The titer of PBS treated or phenol extract treated Clq was 512.

Phenol extracts of the sodium chloride extract of EATC were serially diluted in PBS and tested for the ability to combine with Clq using immunodiffusion analysis. Six dilutions of the phenol extract were placed in the peripheral wells in an Ouchterlony double diffusion plate with 0.6 percent agarose as the supporting medium. Clq was placed

TABLE 6

Inactivation of Guinea Pig Complement Components in Whole Sera by EATC Extracts

Component Tested	Time of Treatment	Number of Effective Molecules in System	Number of Effective Molecules Inactivated
C1	15 min/30°C	2×10^{11}	0
C1	18 hr/4°C	5×10^{10}	1×10^{10}
C2	15 min/30°C	2×10^{11}	0
C2	18 hr/4°C	8×10^{10}	1.5×10^{10}
C4	15 min/30°C	1×10^{10}	0
C3	15 min/30°C	3×10^{10}	0

in the center well. The plate was incubated for two days at room temperature and then for an additional two days at 4°C. Precipitation bands were visible only after 48 hours' incubation at room temperature. The incubation at 4°C was used to intensify the bands. The Ouchterlony plate was photographed and the precipitation pattern observed is shown in Figure 25.

Inhibition of immune hemolysis by RNA from other sources. Escherchia coli RNA obtained from Dr. James Preston (Department of Microbiology, University of Florida) and yeast RNA obtained from Sigma Chemical Company, St. Louis, Missouri were resuspended in PBS, serially diluted, and titrated for inhibitory activity using the immune hemolysis inhibition test. Figure 26 shows the inhibitory activity of E. coli RNA on complement mediated lysis of EA while the inhibitory activity of yeast RNA in shown in Figure 27. The inhibitory activity of the E. coli RNA preparation was approximately 60-fold greater than the commercial yeast RNA preparation.

Sucrose gradient centrifugation of E. coli RNA and of the partially purified tumor cell extracts. The phenol extracted inhibitory material obtained from a crude PBS extract of EATC and E. coli RNA were adjusted to give the same optical density at 260nm. The samples were applied to a preformed sucrose gradient (5 percent to 20 percent w/v sucrose) and centrifuged as previoysly described. After centrifugation, the gradient was monitored at a wavelength

Figure 25. Precipitation of tumor cell extract by human Clq. The center well contains human Clq. The wells shown by the letters A through F, represent dilutions of a partially purified extract obtained by extracting the crude PBS extract from EATC with phenol: well A, 1:2 dilution; well B, 1:4 dilution; well C, 1:8 dilution; well D, 1:16 dilution; well E, 1:32 dilution; well F, 1:64 dilution.

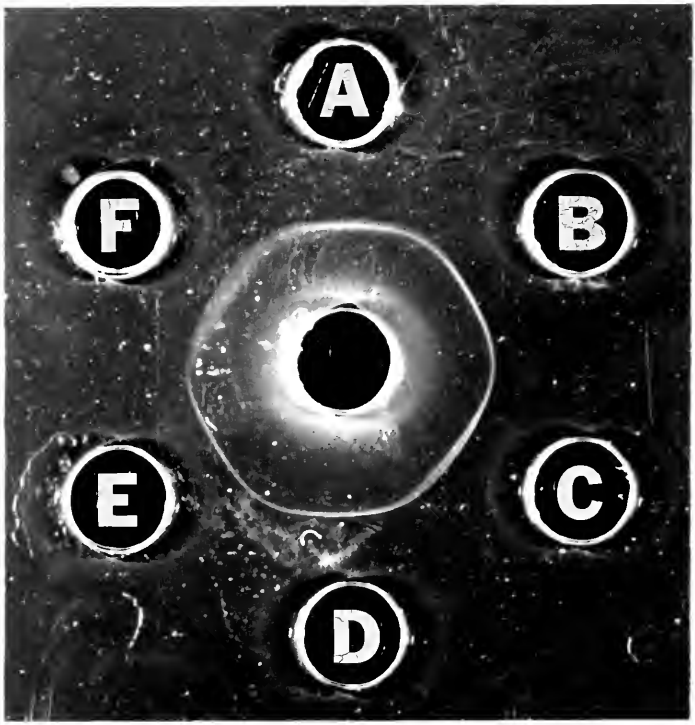

A
B
C
D
E
F

Figure 26. Inhibition of guinea pig complement lysis of EA by an RNA preparation from E. coli.

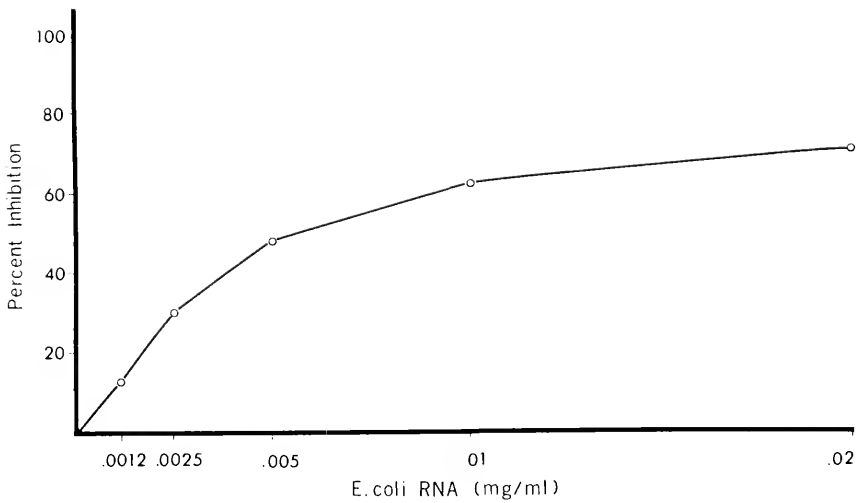
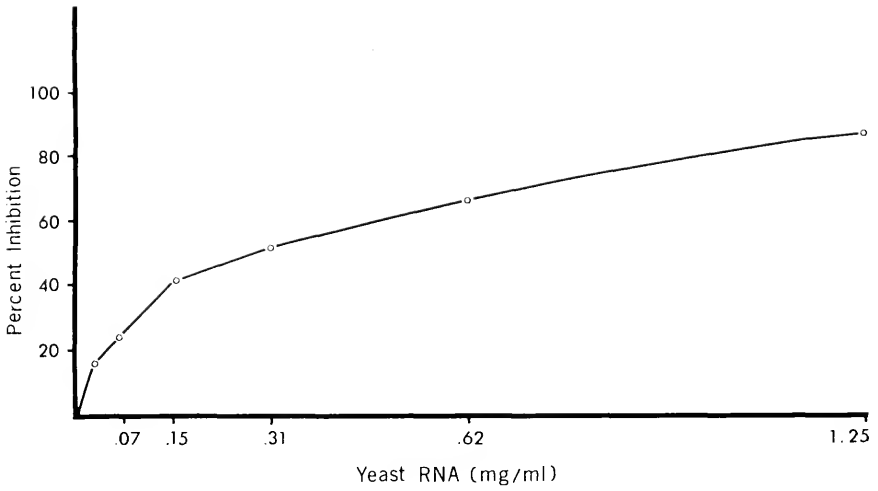

100
80
60
40
20
Percent Inhibition
.0012
.0025
.005
01
.02
E.coli RNA (mg/ml)

Figure 27. Inhibition of guinea pig complement lysis of EA by yeast RNA.


Percent Inhibition
100
80
60
40
20
.07
.15
.31
.62
1.25
Yeast RNA (mg/ml)

of 260nm, fractions were collected, and assayed for inhibitory activity. One drop of each fraction was added to a test tube containing 0.15 ml of $DGVB^{++}$, 0.2 ml of EA at a concentration of 1 X 10^8/ml,and 0.6 ml of diluted guinea pig complement. A control consisted of assaying unloaded sucrose gradient in the same manner. Figure 28 shows the optical density and inhibitory profiles of the phenol extracted tumor material. Figure 29 shows the optical density and inhibitory profiles of the E. coli preparation. As can be seen from Figure 28, the phenol extracted material is heterogeneous with respect to size. The inhibitory activity of the phenol extract seems to be greater than the E. coli preparation. Although the fractions (15 through 17) for E. coli had optical densities at 260nm of about 0.3 to 0.4, they were capable of causing only 60 to 70 percent inhibition while the same fractions for the phenol extracted material, with an optical density at 260nm of about 0.1, were capable of causing 100 percent inhibition of complement mediated lysis of EA.

Viability of EATC and release of complement inhibitory material at 4°C. Ehrlich ascites tumor cells were incubated at 4°C with PBS to determine the length of viability of the cells under these conditions, and to follow the release of complement inhibitory material with time as described in Materials and Methods. Table 7 shows that after 2 hours' incubation, the maximun amount of inhibitory material was obtained with only a 21 percent drop in cell viability.

Figure 28. Sucrose gradient centrifugation of phenol extracted inhibitory material. Fractions collected from the bottom to the top of the gradient. (Fraction 1 = bottom of the gradient.)

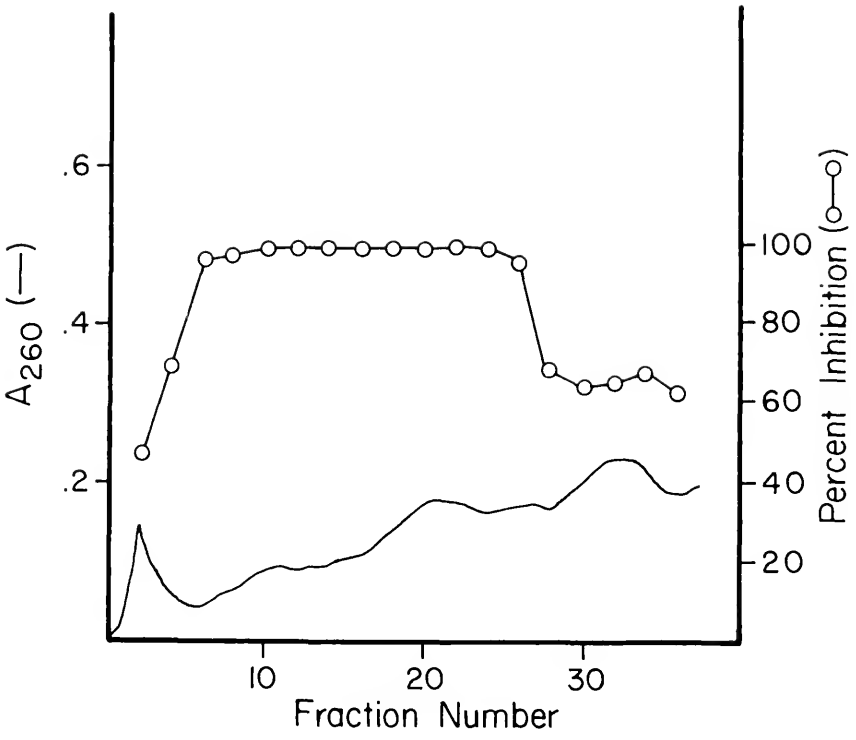

.6
.4
.2
A_{260} (—)
100
80
60
40
20
Percent Inhibition (o—o)
10
20
30
Fraction Number

Figure 29. Sucrose gradient centrifugation of E. coli RNA. Fractions collected from the bottom to the top of the gradient. (Fraction 1 = bottom of the gradient).

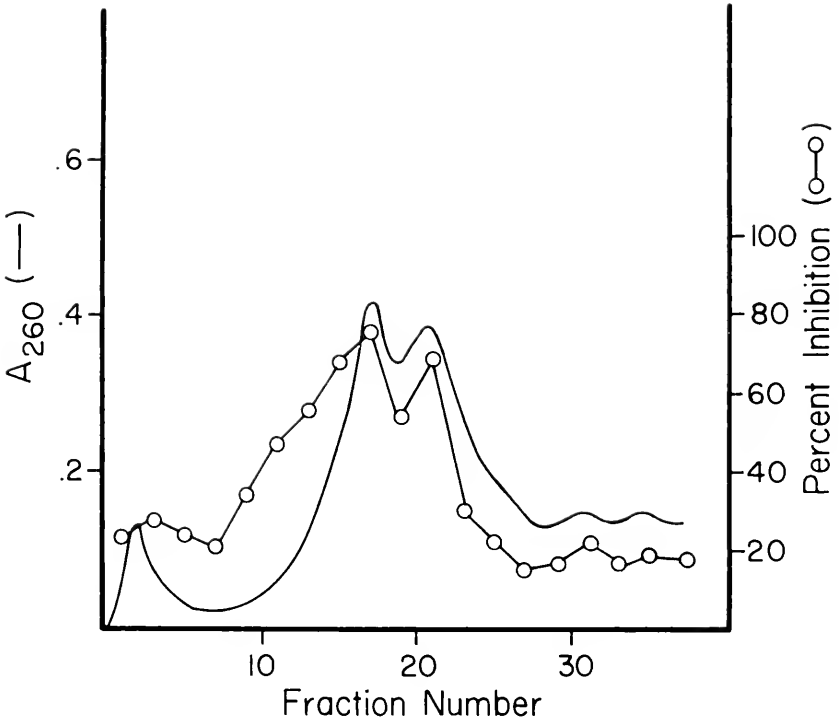

.6
.4
.2
A_{260} (—)
100
80
60
40
20
Percent Inhibition (o—o)
10
20
30
Fraction Number

TABLE 7

Release of Complement Inhibitory Substances from EATC with Time at 4°C

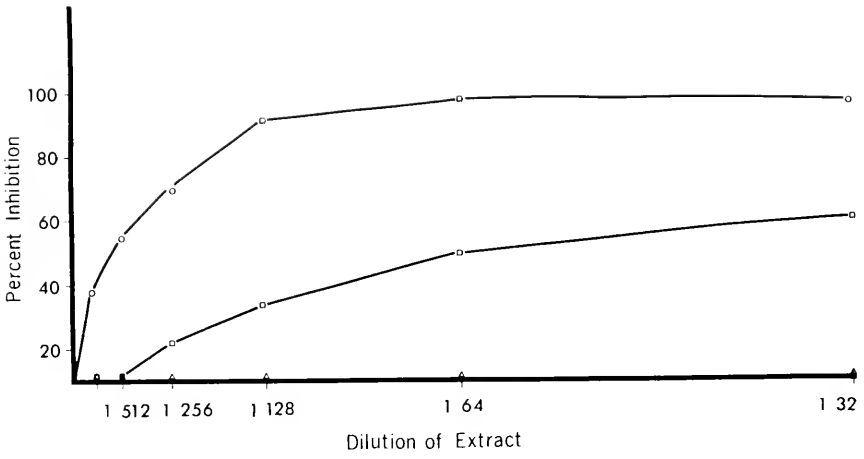
Sample Time (min)	50 Percent Inhibitory Titer[a]	Percent Viability
0	---	88
60	350	67
120	512	62
210	512	61
540	512	51
720	512	45
1400	512	34
1700	256	26

a) Reciprocal of the dilution capable of causing 50 percent inhibition of complement dependent lysis of EA as compared to PBS controls.

Additional incubation did not increase the amount of inhibitory material obrained, but the viability of the cells decreased. The cell count at each sampling time was approximately 1 X 10^9/ml. The failure to obtain an increase in the inhibitory titer with time may be a reflection of the degradation of some of the material by ribonuclease, or it may indicate that viable cells are elaborating inhibitory RNA into the fluid phase.

Inhibition of complement by PBS extracts of normal liver and spleen cells. Normal tissue cells were extracted with PBS to determine if a complement inhibitory substance could be released from these cells under the same conditions used for extraction of EATC. One volume of packed washed liver and spleen cells obtained from non-tumor bearing ICR mice and one volume of packed washed EATC were each shaken overnight at 4°C with three volumes of PBS. The cells were centrifuged down at 500G for 10 min. The supernatents were then centrifuged at 10,000G for 30 min and the remaining supernatent fluids were assayed for inhibitory activity. Figure 30 shows that PBS extracts from spleen cells show no inhibitory activity at the dilutions tested. The liver extract caused 50 percent inhibition of complement mediated lysis of EA at about a 1:64 dilution and the PBS extract of EATC was capable of causing 50 percent inhibition at about a 1:512 dilution. All extracts were adjusted to an optical density of 1.0 at a wavelength of 260nm before titration.

Figure 30. Inhibition of immune hemolysis by dilutions of PBS extracts obtained from EATC (○—○), liver (□—□), and spleen (△—△) cells.


Percent Inhibition
100
80
60
40
20
1 512
1 256
1 128
1 64
1 32
Dilution of Extract

Inhibition of immune hemolysis by phenol extracts of whole normal mouse tissue cells and whole tumor cells. One volume of washed packed normal liver and spleen cells and EATC were each mixed with one volume of PBS and EATC were frozen at -70°C, thawed, and directly extracted with phenol in order to determine if a complement inhibitory material was obtainable by direct extraction of whole cells as described in Materials and Methods. The washed precipitate after phenol extraction and ethanol precipitation was dissolved in two volumes of PBS and dialyzed overnight against 200 volumes of PBS. The extracts were titered for inhibitory activity in the immune hemolysis inhibition test. Table 8 shows that more inhibitory material was extractable from EATC than from liver or spleen cells.

The extracts were adjusted to an optical density at 260nm of 1.0 and 0.2 ml of each extract was layered on a preformed sucrose gradient (5 percent to 20 percent w/v sucrose) and centrifuged. The gradient was monitored at 260nm and fractions were collected as described previously. One volume (0.1 ml) of each fraction was added to a test tube and 0.1 ml of $DGVB^{++}$, 0.2 ml of EA at a concentration of 1 X 10^8/ml, and 0.6 ml of diluted guinea pig complement and the amount of lysis was compared to a blank control gradient. The elution profiles for phenol extracts of whole EATC,liver,and spleen cells are given in Figures 31, 32, and 33 respectively. The elution profiles for the liver and spleen extracts were almost identical

TABLE 8

Extraction of Complement Inhibitory Activity from Spleen, Liver and EAT Cells with Phenol

Cell Type	50 Percent Inhibitory Titer[a]
Spleen	2
Liver	8
EAT	32

a) Reciprocal of the dilution capable of causing 50 percent inhibition of complement dependent lysis of EA as compared to PBS controls.

Figure 31. Sucrose gradient centrifugation of complement inhibitory material obtained by extracting whole tumor cells with phenol. Fractions collected from the bottom to the top of the gradient. (Fraction 1 = bottom of the gradient.)

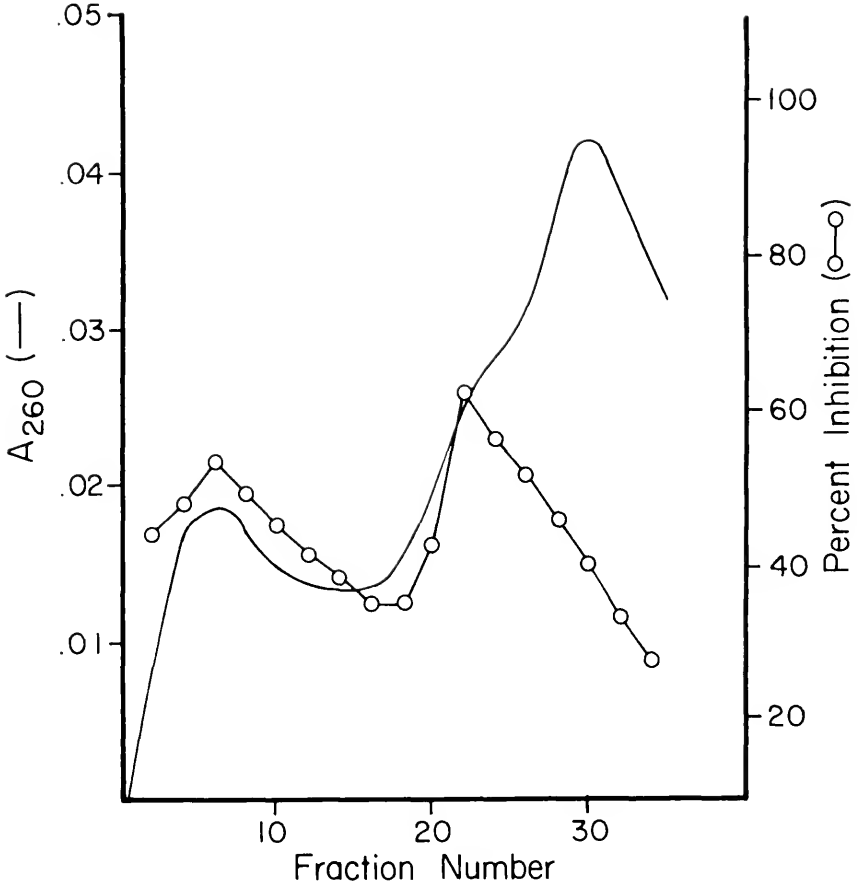

.05
.04
.03
.02
.01
A_{260} (—)
100
80
60
40
20
Percent Inhibition (o—o)
10
20
30
Fraction Number

Figure 32. Sucrose gradient centrifugation of phenol extract of normal mouse liver cells. Fractions collected from the bottom to the top of the gradient. (Fraction 1 = bottom of the gradient.)

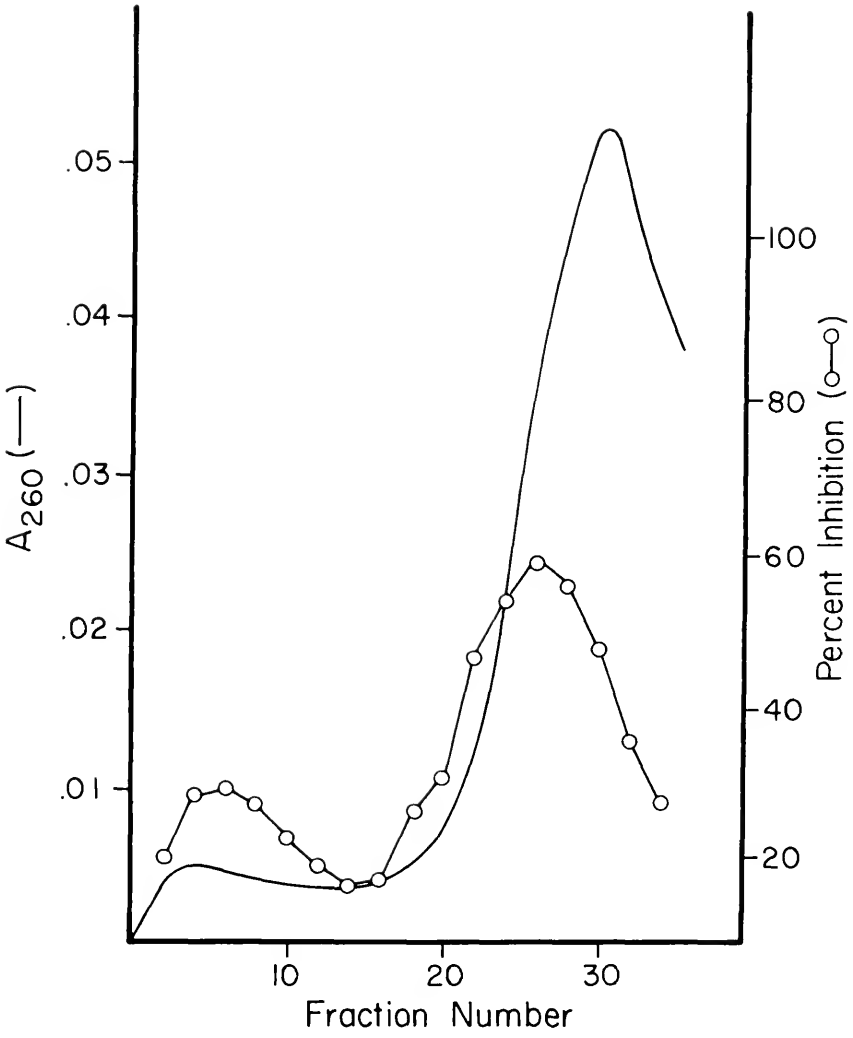
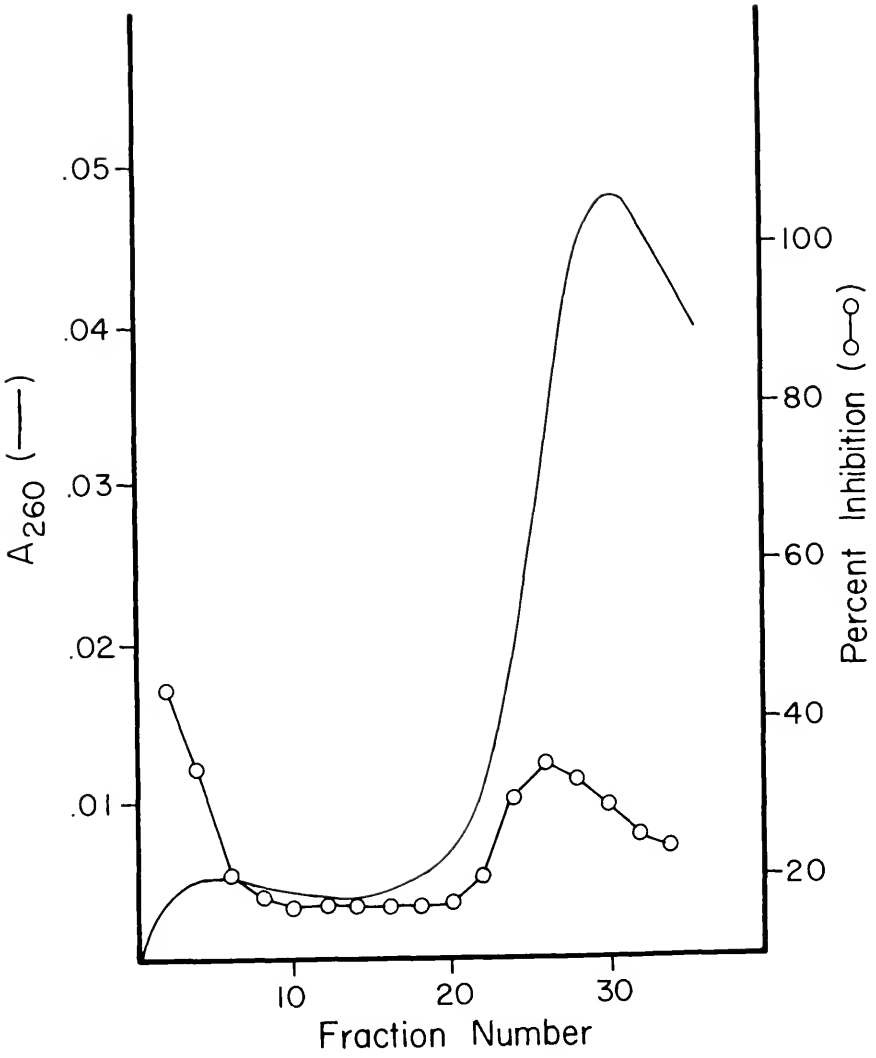

.05
.04
.03
.02
.01
A_{260} (—)
100
80
60
40
20
Percent Inhibition (o—o)
10
20
30
Fraction Number

Figure 33. Sucrose gradient centrifugation of phenol extract of normal mouse spleen cells. Fractions collected from the bottom to the top of the gradient. (Fraction 1 = bottom of the gradient.)


.05
.04
.03
.02
.01
A_{260} (—)
100
80
60
40
20
Percent Inhibition (o—o)
10
20
30
Fraction Number

and showed small molecular weight material at the top of the gradient. The profile for EATC, however, showed that there was some large molecular weight material in the preparation which was more inhibitory than the low molecular weight RNA (based on percent inhibition as a function of A260 of the fractions). This may have accounted for the higher inhibitory titer of the EATC extract compared to those from liver and spleen.

Extraction of a complement inhibitory substance from tumor cell free ascitic fluid. Ehrlich ascites tumor cells were harvested from ICR mice seven days after inoculation of tumor cells. The ascitic cell suspension was centrifuged at 500G for 10 min at)°C. The supernatent was removed and centrifuged at 10,000G for an additional 30 min at 0°C. The second supernatent fluid was removed and extracted with phenol and precipitated with ethanol as described previously. The ethanol precipitated material was redissolved in a volume of PBS equal to the original ascitic fluid volume. The solution was dialyzed overnight versus PBS and titrated for inhibitory activity in the immune hemolysis inhibition test. Table 9 shows the inhibition of complement mediated lysis of EA caused by serial dilutions of the extracted ascitic fluid. The results demonstrated that a complement inhibitory material was present in the ascitic fluid from which the tumor cells were harvested.

TABLE 9

Inhibition of Complement Mediated Lysis of EA by Phenol Extracts of Ascitic Fluid from Ehrlich Ascites Tumor Bearing Mice

Dilution of Extract	Percent Inhibition
1:2	75
1:4	53
1:8	42
1:16	30
1:32	25
1:64	4

Extraction of a complement inhibitory substance from P815 and EL4 tumor cells. The tumor lines P815 and EL4 were used to see if a complement inhibitory substance was extractable from tumor cells other than Ehrlich ascites. P815 and EL4 tumor cells were harvested and washed and extracted as outlined in Materials and Methods. Table 10 shows that an inhibitory substance was extractable from P815 and EL4 tumor cells. P815 gave consistently higher yields of inhibitory material. Since only one extraction method was used, the yield of inhibitory material might be a reflection of the type of procedures used for extraction and not a measure of the amount of inhibitory substance present in these two tumor lines.

Cytotoxicity of EATC in the presence of ribonuclease. The resistance of tumor to the action of antibody and complement might be due to the presence of RNA on the tumor cell surface or the release of RNA into the surrounding medium. Since ribonuclease treatment destroyed the inhibitory activity of the tumor cell extract, the ability of rabbit antibody and guinea pig complement to cause EATC lysis was tested both in the presence and absence of ribonuclease. As can be seen from Table 11, treatment of the cells before incubation with an antibody and complement did not increase the amount of cells killed and there was even a slight decrease in killing as compared to controls. Also, addition of ribonuclease to the reaction tube which contained complement did not have any significant effect on

TABLE 10

Extraction of Complement Inhibitory Material from P815 and EL4 Tumor Cells

Cell Type	Experiment Number	50 Percent Inhibitory Titer[a]
P815	1	40
P815	2	80
EL4	1	5
EL4	2	20

a) Reciprocal of the dilution capable of causing 50 percent inhibition of complement dependent lysis of EA as compared to PBS controls.

TABLE 11

Inhibition of Cytoxicity of EATC with Ribonuclease

Experiment Number	Percent Cell Death	
	Treated	Control
1[a]	66 ± 6	70 ± 4
1	22 ± 2	29 ± 7
2[b]	66 ± 8	74 ± 3
2	25 ± 3	24 ± 2

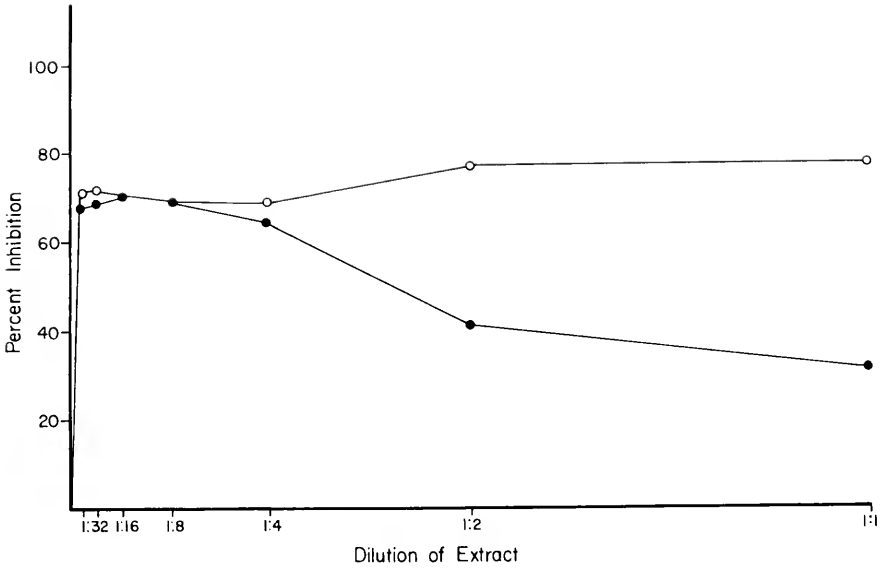
a) EATC treated with Ribonuclease and washed prior to cytotoxicity test.

b) Ribonuclease added with other reactants in test.

the ability of complement to kill EATC. The failure of ribonuclease to influence complement mediated tumor cell killing in the experiment described might be due to the failure of RNA to be expressed or released by the tumor cells under the conditions used.

Inhibition of EATC cytotoxicity by EATC extracts. Extracts from EATC were tested for the ability to inhibit killing of the tumor cells by antibody and complement. Crude EATC extracts and partially purified inhibitory material obtained by extracting the crude extract with phenol were incubated with EATC, sensitized with antibody, and complement. Controls consisted of adding PBS instead of the tumor cell extract. The amount of inhibition caused by the tumor cell extracts is shown in Figure 34. The cytotoxicity of the control cells was approximately 50 percent. The crude tumor cell extract showed a consistent inhibitory activity in the dilutions tested. However, the phenol extracted material demonstrated more inhibitory activity at the lower concentrations than at with higher levels.

Figure 34. Inhibition of EATC cytotoxicity by EATC extracts. The open circles (O—O) represent the inhibition of complement mediated cytotoxicity of EATC by the sodium chloride extracted inhibitory substance. The inhibition of cytotoxicity of EATC by partially purified, phenol extracted inhibitory material is given by the closed circles (●—●). The cytotoxicity of control cells was approximately 50 percent.


100
80
60
40
20
Percent Inhibition
1:32
1:16
1:8
1:4
1:2
1:1
Dilution of Extract

DISCUSSION

Tumors vary with regard to susceptibility to antibody and complement mediated cell damage (62). The resistance of some tumor cells to the lytic action of antibody and complement may be due to many factors such as low antigen density on the tumor cells, inaccessibility of the humoral factors to the tumor, stimulation of noncomplement fixing antibodies by the tumor, or the presence of complement inhibitors in the sera or associated with the tumor itself. Complement inhibitors may also play a role in cell mediated immune reactions. For example, complement components can enhance phagocytosis (105). Also, the complement component fragments C3a and C5a have chemotactic properties for polymorphonuclear leukocytes and other phagocytic cells (33,42). The possession and release of a complement inhibitory substance may enhance the survival of that tumor in the face of immunological challenge because the complement system would be blocked from reacting against the tumor.

The results of this investigation show that Ehrlich ascites tumor cells possess some factor or factors that inhibit the lytic action of complement, from a number of different animal sources, on sensitized sheep erythrocytes.

The inhibition of complement is apparently not due to complement fixation by tumor cell extracts. The crude, PBS extract used in the initial studies did not affect the titration of antibody to sheep red cells indicating that there was no cross reaction between sheep erythrocyte antigens and EATC extracts. The results of the $EAC\overline{1\overline{42}}$ decay experiments indicated that the tumor cell extract did not alter the decay rate of $SAC\overline{1\overline{42}}$. This differentiates the inhibitory activity found here from that isolated from human erythrocytes, since extracts from human erythrocyte stromata have been shown to accelerate the decay of the $EAC\overline{1\overline{42}}$ intermediate (87). However, the extent of lysis of the $EAC\overline{1\overline{42}}$ incubated with EATC extracts was less than that of controls. The extract did not affect the stability of $EAC\overline{1}4$ but treatment of the cells with the extract caused an impairment of the cells to undergo lysis. No intermediate steps after $EAC\overline{1\overline{42}}$ were inhibited by EATC extracts. Therefore, it was obvious that treatment of the cellular intermediates in the complement cascade with EATC extracts affected one or more of the early steps in the complement sequence while having no apparent effect on the terminal intermediates.

Experiments conducted to define the site of action of the crude extract showed that treatment of EA with the tumor cell extract increased the ability of EA to take up C1. Although more C1 was taken up by EA treated with tumor cell extract, cells were inhibited from lysing (Table 1). Since

EA were not inhibited in the ability to take up Cl, the action of the tumor cell extract could interfere with the reactivity of Cl once it was on the cell surface. In support of this, it was shown that EAC$\bar{1}$ treated with the tumor extract were less efficient in consuming C4 than were untreated control cells. Additional evidence for EATC extract inhibiting bound Cl was obtained when EAC$\bar{1}$4 were treated with extract. Treated EAC$\bar{1}$4 had a longer Tmax than did untreated control EAC$\bar{1}$4. However, the crude tumor cell extract did not inactivate Cl in the fluid phase under the conditions tested. These results support the concept that the crude extract inhibits complement activity by interfering with cell bound reactivity.

The inhibitory action of the crude tumor cell extract may be such that the inhibitory material is capable of binding to antibodies or directly to the cell surface. On the other hand, the tumor extract might react with the antibody coated erythrocyte in such a way that it alters the cell surface without binding. This alteration might not interfere with the binding of Cl, but once Cl is attached, its reactivity is inhibited. Another possible explanation is that there is more than one inhibitory substance in the crude extract. Other inhibitory substances, distinct from the Cl inactivating substance, may be responsible for inhibiting the other intermediates tested. Alternatively, the Cl inactivating substance may be coupled to a molecule that permits the extract to bind to sensitized sheep erythrocytes.

Since it was difficult to choose between the alternatives suggested, the crude tumor cell extract was subjected to purification and the mechanism of action of the partially purified extract was again examined. The inhibitory material obtained after phenol extraction of the crude extract was capable of interfering with the lysis of only two intermediates, EAC$\overline{1}$ and EAC$\overline{1}$4. These results pointed more directly at C$\overline{1}$ as the site of action. It is unclear, however, why only the above intermediates were affected by the purified inhibitor since multiple inhibitors were not found. The phenol extract prevented transfer of active C1 from treated EAC$\overline{1}$. However, partially purified extract was incapable of inactivating fluid phase C1, C2, C4, or C3 in whole sera and purified C1, and C2 under the same conditions that were used to inactivate C1 on EAC$\overline{1}$ cells (30°C/15 min). Partial inactivation of purified C1, and C1 and C2 in whole guinea pig serum was obtained only after prolonged incubation with extract at 4°C. Evidence was obtained which indicated that the active inhibitor in the purified preparations was RNA, a polyanion. If the extract acts like other polyanions, such as DNA, then the inactivation of C2 might be a result of the activation of C1 to C$\overline{1}$ which then cleaves and inactivates C2. However, the tumor cell extract may also have a direct effect on C2 in whole serum. The second alternative seems unlikely since there was no inactivation of C2 on EAC$\overline{142}$ by the phenol extract.

Certain polyanions have been shown to inactivate C1 by binding to the C1q component of the molecule (18,106).

The phenol extract was able to cause a visible precipitation of Clq as shown in Figure 25, but the reaction only became visible after 2 days incubation. The tumor cell extract, however, did not inactivate Cl in the fluid phase in the routine reaction procedure (30°C/15 min) but could inactivate Cl on the cell surface completely under these conditions. Therefore the findings of this research suggest that the tumor extract might inhibit complement by affecting Cl, but primarily on the cell surface. The failure of EATC extract to inactivate $C\bar{1}$ in the fluid phase might be a result of inhibitory material forming weak interactions with $C\bar{1}$ that are easily dissociated or the failure of that interaction to activate Cl. There have been two previous reports concerning the interaction of RNA with complement. In the first report, Yachnin showed that RNA could not inhibit complement mediated lysis of sheep EA (21). However, a later report by Agnello et al. showed that RNA and several other polyanions could complex with Clq (17). The very effective inactivation of $C\bar{1}$ at the cell surface by the tumor cell inhibitor may be a consequence of an altered Cl molecule. The configuration of the Cl molecule could change after binding to antibody on the cell surface. Such a change could expose additional binding sites on the Cl molecule so that the inhibitor can react more efficiently. Another alternative is that partially purified extract may react with the altered cell surface of $EAC\bar{1}$ in such a manner that prevents Cl to function normally. Still another possibility is that the inhibitor could react at the C2 reactive site of $C\bar{1}$ since $EAC\bar{1}4$ cells are inhibited but

EAC$\overline{142}$ cells are not. The inhibitory material could also directly displace Cl from the cell surface. However, no effective Cl molecules could be transferred from EAC$\bar{1}$ after treatment with the tumor extract. Perhaps the Cl was bound by the inhibitor and removed from the cell as an inactive complex. The inhibitory substance could also have inactivated C$\bar{1}$ on the surface of the cell without removing the molecule. Further work is warrented to decide between the several alternatives that have been mentioned in order to pinpoint the precise mechanism of action of this inhibition.

Recently Loos et al. have shown that certain polyanions can inhibit C4 and C2 binding to the Cls part of C$\bar{1}$ (107). This report adds new dimensions as to the site of binding of polyanions of C$\bar{1}$ and the mechanism of inhibition of Cl.

The first attempts at purification using ammonium sulfate fractionation, DEAE chromatography, and gel filtration resulted in poor yields. The gel filtration data indicated, however, that the inhibitory substance had a molecular weight between 10^5 and 1.5 X 10^6 daltons. The material was not retained by G-200 sephadex which excludes proteins of molecular weight 800,000 and dextrans of molecular weight 200,000 (described by the manufacturer, Pharmacia Fine Chemicals, Piscataway, New Jersey). The inhibitory material was retained by Bio-Gel A-1.5m which separates proteins of molecular weight of 20,000 to 1.5 X 10^6. The molecular weight ranges for Bio-Gel A-1.5m are those given by the manufacturer (Bio-Rad Laboratories, Richmond, California). More effective partial purification

of the tumor inhibitory material was accomplished using a combination of phenol extraction and DEAE chromatography. Based on the optical density ratio of the partially purified inhibitor at wavelengths of 280nm/260nm, the purified material appeared to be a nucleic acid. DEAE chromatography of the crude extract followed by phenol extraction of the inhibitory fractions resulted in the recovery of a material that was essentially 100 percent nucleic acid. The active inhibitory material bound very strongly to DEAE which suggested it had a high net negative charge. The material was sensitive to ribonuclease and the optical density ratio of 280nm/260nm was characteristic of a nucleic acid. On the basis of these findings, the active portion of the tumor cell extract appeared to be associated with a polyanion, namely RNA.

Sucrose gradient centrifugation of the phenol extracted material showed that the RNA obtained was heterogeneous in terms of molecular weight when compared to purified E. coli RNA. The sucrose gradient analysis of E. coli RNA and the RNA from EATC showed that the RNA from the tumor extract had a somewhat higher inhibitory activity than the E. coli fractionated polyribonucleotide. Yeast RNA was also shown to inhibit guinea pig complement. Inhibition was not strictly restricted to a particular size of RNA; however, the smaller molecular weight material seemed to be somewhat less effective. These findings are in contrast to the report by Yachnin that RNA was incapable of inhibiting guinea pig or human complement (21).

Normal tissue cells were compared to EATC in an attempt to see if normal cells possess a complement inhibitory activity which can be extracted under the mild conditions used for extraction of EATC. PBS extracts from EATC were consistently more inhibitory than were extracts of normal liver and spleen cells. Direct extraction of intact cells with phenol again showed that extracts of tumor cells possessed more inhibitory activity than extracts of normal liver and spleen cells when the extracts were all adjusted to the same relative absorbance at 260nm before testing. Sucrose gradient centrifugation of the phenol extracts showed that the liver and spleen RNA extracts consisted mostly of small molecular weight RNA. The RNA extracted from intact EATC, however, was largely a high molecular weight variety. Since the cells were frozen and thawed before extraction with phenol, the RNA of spleen and liver cells could have been degraded before extraction with cell associated ribonuclease. In the case of the Ehrlich ascites tumor extract, the cells may possess a much lower amount of endogenous ribonuclease, thereby less RNA is degraded. However, there exists the alternative possibility that EATC possess a certain class of RNA that is protected from degradation by ribonuclease.

This study has also shown that a complement inhibitory activity can be extracted from two other tumor lines, P815 and EL4. Therefore, this kind of activity is not restricted to a single type of tumor. Furthermore, phenol extraction

of the ascitic fluid from tumor bearing mice demonstrated the existance of an extractable nucleic acid that was capable of inhibiting guinea pig complement. Finally, the tumor cell extract derived complement inhibitor was capable of inhibiting complement dependent killing of Ehrlich ascites rumor cells, as well as inhibiting lysis of sheep erythrocytes.

Although extracts from EATC have been shown to inhibit complement in vitro, the role of this material in vivo is uncertain. Further work is also needed to determine which class of RNA is responsible for the inhibition. The possibility exists that a unique class of RNA associated with the tumor cell may be responsible for the inhibitory activity in vivo. The ease of extracting the inhibitory material with PBS also suggests that the material may be associated at the cell surface. In support of this idea, Mayhew has shown that treatment of Ehrlich ascites tumor cells with ribonuclease altered their electrophoretic mobility. He showed that this was not due simply to charge neutralization after nonspecific adsorption of the ribonuclease (108). Shapot and Davidova have suggested that RNA may be an integral part of animal plasma cell membranes (109). They suggest that a certain class of RNA is present in the plasma membrane and that it may act as a structural component.

Tumor cells with a high metabolic rate could have a high rate of turnover of RNA which could then allow some of the RNA to be excreted into the surrounding growth medium.

Reports of the effects of polyanions and ribonuclease on tumor growth are confusing. Ledoux has reported that injection of ribonuclease into mice with ascites tumors significantly increased the survival time of treated mice (110). Administration of the polyanion dextron sulfate, to AKR mice, led to an acceleration of leukaemia development, whereas two polycations, DEAE-dextran and polybrene, increased the mean survival time of the mice (111). Dextran sulfate has been shown to be anticomplementary (21). Maekawa and Kushibe have shown, however, that growth of Ehrlich ascites tumor cells could be inhibited by injecting RNA along with the tumor (112). In this case, addition of enough RNA, beyond the amount that cell is capable of producing, could alter the cell membrane to such an extent that new antigen sites are exposed. Enough RNA present could allow direct activation of complement at the cell surface so that activated complement components may then attach to the cell and form a lytic site. Perhaps fluid phase activation could result in "innocent bystander" lysis also.

The failure of complement to participate in rejection of a number of tumors has not been adequately explained. Work by Cooper and coworkers have shown that Maloney virus induced tumors are only susceptible to complement mediated killing during the G1 phase of the cell cycle (77,78). Recently Ohanian has proposed that resistance of some tumors may not be a reflection of the amount of complement fixed to the cell, but may be due to an intrinsic property of the

tumor cell that depends on the manner in which C1, C4, and C2 are fixed to the cell surface (113). The reason for the lack of tumor cytotoxicity by complement is not apparently due to the inability of complement to invade the tumor mass. Irie et al. have shown that tumors growing in vivo possess bound complement components on their surface (114).

A complement inhibitor either associated with or released from tumor cells could greatly enhance tumor survival. Normal tissue cells may possess complement inhibitory substances as in the case of human erythrocytes (87). If cells become neoplastic the cell associated inhibitor could act to prevent cell destruction. RNA may also play a similar and significant role in tumor survival. The active release of RNA by the tumor or possession of RNA in the membrane of animal cells would give the tumor protection from cell destruction by complement. Although this report only suggests that tumor cell associated RNA might contribute to tumor survival in an immune host, the possibility does exist, and further investigations are hopefully warrented from these findings.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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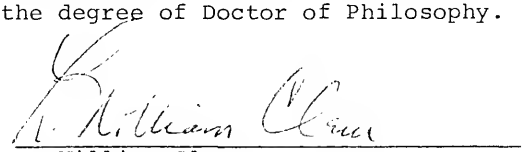

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June, 1975

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